Federation Proceedings

VOLUME 18 *December 1959* NUMBER 4

CONTENTS

SYMPOSIUM ON BIOCHEMICAL ASPECTS OF CELL STRUCTURE AND FUNCTION

Genetic Activity of Deoxyribonucleic Acid in the Reconstitution of Biosynthetic Pathways. *John Spizizen* 957

SYMPOSIUM ON TOPICS OF CURRENT INTEREST IN BIOCHEMISTRY

Chemistry of Bacterial Cell Walls. *Friedrich Zilliken* 966

Photosynthetic Phosphorylation. *Andre T. Jagendorf* 974

Interconversions of Hexoses. *Saul Roseman* 984

Role of Lipides in the Mitochondrial Electron Transport System. *D. E. Green and R. L. Lester* 987

SYMPOSIUM ON AUTONOMIC PHARMACOLOGY

Non-nicotinic Ganglion-Stimulating Substances. *Ullrich Trendelenburg* 1001

Enzymic Oxidation of Pharmacologically Active Amines. *Paul Hagen and Norman Weiner* 1005

SYMPOSIUM ON TOXICOLOGY

Introduction. *Harold C. Hodge* 1013

Mechanism of Action of Carbon Tetrachloride. *J. D. Judah and K. R. Rees* 1013

DISCUSSION. *Theodore M. Brody* 1017

Recent Studies of Organic Phosphate Poisoning. *J. H. Wills* 1020

Cell Membrane as Site of Action of Heavy Metals. *Aser Rothstein* 1026

DISCUSSION. *T. W. Clarkson* 1036

SYMPOSIUM ON FERTILITY-CONTROLLING STEROIDS

Introduction. *Hudson Hoagland* 1039

Biological Effects of Some Steroids With Progestational Activity. *Victor A. Drill* 1040

DISCUSSION. *Daniel A. McGinty* 1048

Effects of Certain 19-Nor Steroids on Reproductive Processes and Fertility. *Gregory Pincus, John Rock, M. C. Chang and C. R. Garcia* 1051

DISCUSSION. *Robert B. Greenblatt* 1055

REPLY TO DISCUSSION. *Gregory Pincus*

Effects of Progesterone and Synthetic Progestins on the Reproductive Physiology of Normal Men. *Carl G. Heller, Donald J. Moore, C. Alvin Paulsen, Warren O. Nelson and William M. Laidlaw* 1057

DISCUSSION. *Warren O. Nelson* 1065

Use of New Progestational Hormones in Gynecology and Obstetrics. *A. E. Rakoff* 1066

DISCUSSION. *Herbert S. Kupperman* 1071

(Contents continued)

SYMPOSIUM ON EFFECTS OF HIGH CALCIUM INTAKES

Introduction. *Olaf Mickelsen* 1075
Calcium Intake of Children and Adults. *Margaret A. Ohlson and Genevieve Stearns* 1076
Factors Influencing Calcium Absorption. *Harold E. Harrison* 1085
Effects of High Calcium Intakes on Renal Function. *Philip H. Henneman* 1093
Effects of High Calcium Intakes on Urine in Animals. *C. W. Vermeulen* 1096
DISCUSSION. *Robert S. Harris* 1100
Effects of High Calcium Intakes on Urine in Human Beings. *William H. Boyce and J. Stanton King, Jr.* 1102
Effects of High Calcium Intakes on Bones, Blood and Soft Tissue; Relationship of Calcium Intake to Balance in Osteoporosis. *G. Donald Whedon* 1112
Effects of High Calcium Intakes on the Absorption of Other Nutrients. *George K. Davis* 1119
DISCUSSION. *D. Mark Hegsted* 1123

SYMPOSIUM ON PROTEIN REQUIREMENT AND ITS ASSESSMENT IN MAN

Introduction. *Paul György* 1125
Nitrogen Content of the Body and Its Relation to Retention and Loss of Nitrogen. *W. M. Wallace* 1125
Protein Requirement in Man. *D. Mark Hegsted* 1130
Amino Acid Balance and Protein Requirement. *A. E. Harper and U. S. Kumta* 1136
Protein Nutrition and Enzyme Changes in Man. *J. C. Waterlow* 1143
Protein Requirements in Injury and Certain Acute and Chronic Diseases. *Stanley M. Levenson and Donald M. Watkin* 1155
Protein, Calories and Life Expectancy. *M. H. Ross* 1190
Protein Malnutrition and Infection. *Nevin S. Scrimshaw* 1207
PANEL DISCUSSION 1212

SYMPOSIUM ON BIOLOGICAL RHYTHMS

Photobiology and Biochemistry of Circadian Rhythms in Non-Photosynthesizing Cells. *Charles F. Ehret* 1232

SYMPOSIUM ON LIFE IN SPACE

Introduction. *Wallace O. Fenn* 1241
Radiation and Life in Space. *Cornelius A. Tobias* 1242
Some Metabolic Problems of Space Flight. *Hans G. Clamann* 1249
Progress Toward Space Flight. *Paul A. Campbell* 1255

NEWS AND NOTES xlviii

FEDERATION ANNUAL MEETING—CHICAGO 1960 lvi

AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Biochemical Aspects of Cell Structure and Function[1]

Chairman: HARLAND G. WOOD

GENETIC ACTIVITY OF DEOXYRIBONUCLEIC ACID IN THE RECONSTITUTION OF BIOSYNTHETIC PATHWAYS

JOHN SPIZIZEN

Department of Microbiology, Western Reserve University, Cleveland, Ohio

THE EASE WITH WHICH genetic markers may be obtained and assayed makes microbial cells excellent material for the study of genetic phenomena on the biological and molecular level. Heritable traits such as nutritional requirements, antibiotic resistance or sensitivity, resistance or sensitivity to infection by viruses are readily obtainable for any strain by mutagenic action. Simple selection methods have been developed—but most important, the quantitative detection of such 'marked' strains can be performed with ease and efficiency in microbial experiments.

PROBLEM OF GENETIC STUDY ON THE MOLECULAR LEVEL

By means of such methods, three main types of genetic transfer systems in bacterial populations have been revealed. In the first system, revealed by the brilliant studies of Tatum and Lederberg (1), exchange of genetic material occurs by means of a sexual process in which the mating of two types is involved. The transfer of nutritional markers as well as antibiotic sensitivity markers can be readily demonstrated. By using donor cells which can be killed by particular antibiotics and by certain viruses after donating the genetic material, it is possible to follow the kinetics of formation of new systems in the recipient cells which are resistant to the same antibiotics and viruses (2).

A second method of genetic transfer is that which is mediated by bacteriophages of the nonvirulent type. This process which is akin to phage 'mating' with a recipient cell is called transduction (3). In this process, genetic material from a previous host becomes part of the temperate phage particle and when such a particle injects its deoxyribonucleic acid (DNA) into a cell, it carries some of the genetic information from its former environment.

A third method of genetic exchange is one in which genetic molecules in the form of DNA solutions isolated from a donor strain can enter some of the bacterial cells after simple mixing, and thus transmit specific genetic traits (4). This is often spoken of as 'bacterial transformation'.

Although these methods of genetic exchange involve different biological states of DNA, direct proof for the involvement of molecular DNA as gene material has come only from a study of the transformation process. That DNA was at least a part of the genetic material was early inferred from direct cytological observations of the activity of the nucleus following ovum fertilization. The nucleus was shown to contain the bulk of cellular DNA by Feulgen staining and isolation procedures. The classical studies of Morgan (5) and his collaborators on the correlation of cytological and genetic evidence in studies of the salivary gland chromosomes in Drosophila gave added weight to the thesis that DNA was directly associated with gene activity.

[1] Atlantic City, New Jersey, April 13, 1959.

However it was Avery, MacLeod and McCarty in 1944 (4) who demonstrated that isolated DNA could induce a genetic alteration in the form of a specific capsular antigen change in pneumococcal cells. In these experiments, highly polymerized DNA isolated from a pneumococcus characterized by the presence of Type III polysaccharide, after mixing with actively growing Type II organisms in the rough form would induce the formation of smooth Type III pneumococci in a small percentage of the population. Proof that DNA was in fact the genetic agent was obtained by the demonstration that purification, particularly the removal of protein, gave active material and that it was extremely sensitive to inactivation by deoxyribonuclease. Criticism could still be made that the DNA might be protecting some nonidentified component of these preparations. However, subsequent work has indicated that this is unlikely (6).

These experiments were extended by other workers to many specific capsular types not only in Pneumococcus but in Hemophilus and Meningococci. In table 1 it may be seen that a number of types of heritable traits can be transferred by DNA preparations. Resistance to drugs, such as antibiotics, has been very useful in transformation studies in Pneumococcus and Hemophilus.

We have recently discovered that the organism, *Bacillus subtilis*, can also be transformed by DNA solutions, not only for drug resistance but also for a number of biochemical markers (12). The latter characters are made possible by the fact that unlike Pneumococcus and the other transformable organisms, *B. subtilis* can grow on simple media containing ammonium salts as the nitrogen source and glucose as the carbon source. It is thus possible by conventional mutating devices, such as ultraviolet light, to induce nutritional blocks of many types as genetic markers. When stable, nutritionally-deficient organisms are mixed with DNA isolated from the wild type strain, a fraction of the culture becomes converted to nutritionally independent organisms which can be readily isolated by plating on minimal medium lacking the nutritional factor formerly required. The conditions under which this occurs are exacting and will be discussed later.

Another type of genetic trait which can be transferred by DNA molecules in solution is the ability to form specific enzymes of the inducible type. This can be shown in Pneumococci (13) and in our new *B. subtilis* system (unpublished results). Before discussing this problem, it will be noted that the formation of a tumor, the crown-gall tumor, in plants has been shown to be induced by the microorganism, *Agrobacterium tumefaciens*. Only certain organisms which we can call virulent are capable of tumor formation and this character has been shown to be transferred via DNA to nonvirulent types. It is of further interest that recent experiments of Thomas and Klein (14) indicate that DNA isolated from the virulent organism can induce crown-gall tumors in plants directly. It will be necessary, however, to show that DNA isolated from the crown-gall tumor cells can continue to initiate the tumor in healthy plants in order to establish that this latter phenomenon is similar to transformation in bacterial organisms.

TABLE 1. GENETIC ACTIVITY OF DNA IN MICROBIAL SYSTEMS

Character Transformed	Microorganism
Capsule containing specific polysaccharides	*D. pneumoniae* (4) *H. influenzae* (7) *N. intracellularis* (8) (*E. coli*) (9)
Resistance to drugs	*D. pneumoniae* (10) *H. influenzae* (11) *B. subtilis**
Nutritional independence	*B. subtilis* (12)
Enzyme-forming ability	*D. pneumoniae* (13) *B. subtilis**
Virulence in plants (tumor-inducing ability)	*A. tumefaciens* (14)

* Spizizen, unpublished results.

We can define the phenomenon of genetic transfer by molecular DNA in microorganisms in the following manner. DNAase-sensitive DNA can be obtained from wild type bacteria which will penetrate certain strains, usually closely related, thereby introducing a new genetic trait. Only a fraction of cells of the recipient culture are so altered; however, the frequency is usually many thousand times greater than spontaneous mutations which might appear. Usually single characters are transmitted to a cell with higher frequency than double or more characters, implying that the genetic entities can be separated. When more than one character is transmitted with high frequency, we can infer that the molecules are 'linked' or combined in some fashion. We will discuss such a case later. In these transfers, the character is permanent, so that the progeny receive it indefinitely. This means that the new DNA replicates, and thus the DNA isolated from the progeny, will transform other cells for this character.

CONVERSION OF BIOCHEMICALLY-BLOCKED STRAINS OF BACILLUS SUBTILIS BY DNA

To illustrate some of the principal features of genetic activity by DNA molecules, some experimental findings in our new *B. subtilis* system will be presented.

A number of mutant strains of *B. subtilis* were employed which were derived by ultraviolet irradiation from wild type organisms, i.e. those able to grown on minimal medium. These are shown in table 2. When DNA isolated from wild type strain 23 was mixed with suspensions of any of these strains under appropriate conditions, nutritionally independent organisms were obtained. The number of organisms which have been transformed by contact with DNA to nutritional independence can be determined by plating on minimal agar and counting the number of colonies which appear after 24 hours' incubation. When two nutritional requirements are present in the mutant, completely independent organisms were obtained by transformation in a much lower frequency than those transformants requiring only one or another of the nutritional compounds. Thus when strain P_1 was used as recipient, conversion to indole-requiring, isoleucine independent organisms occurred with the same frequency (1%) as indole-independent, isoleucine-requiring ones, but completely independent organisms are formed with a much lower frequency (.01%).

The DNA was isolated from the wild type *B. subtilis* in the following manner (12). Packed cells of the strain 23 were crushed in a modified Hughes Press, or protoplasts made by lysozyme treatment in 20% sucrose were lysed in hypotonic saline (unpublished observations). The broken cells were rapidly washed twice in 0.1 M sodium chloride and .05 M sodium citrate to remove protein and some RNA. The insoluble residue was then extracted in the cold with 2 M sodium chloride and after centrifugation the DNA was precipitated from the supernatant liquid by the addition of cold ethanol to a final concentration of 70%. The fibers were resuspended in 2 M sodium chloride solution and then deproteinized by exhaustive chloroform-octanol extractions. A constant proportion of RNA was always found associated with the DNA at this point. This RNA could only be removed by treatment with crystalline ribonuclease (50 γ/ml) and the DNA could then be precipitated free of RNA.

TABLE 2. B. SUBTILIS STRAINS GENETICALLY ALTERED TO NUTRITIONAL INDEPENDENCE BY DNA

(Donor DNA isolated from 'wild type' 23)

Strain	Nutritional Block
166 (Yale)	anthranilic acid
168 (Yale)	indole
160 (Yale)	L-tryptophan
122 (Yale)	nicotinic acid
9 (Guthrie)*	purines
P_1	isoleucine and indole

* Kindly supplied by Dr. R. Guthrie, University of Buffalo.

TABLE 3. ISOLATION OF DNA FROM B. SUBTILIS, 23

Fraction	DNA/ Protein	DNA/ RNA
I. Solution in 2 M NaCl after first alcohol precipitation	0.47	*
II. Exhaustive chloroform-octanol extraction followed by alcohol precipitation and solution in 2 M NaCl	>60	2.0
III. Ribonuclease (50 γ/ml) treatment followed by alcohol precipitation and solution in 2 M NaCl	>60	>60

* At least 2.0 and often greater, depending on the rapidity with which the DNA fibers are removed from the alcohol, since RNA precipitates out after the DNA.

In table 3 are presented the ratios of DNA to protein and DNA to RNA obtained during various steps in the fractionation. It can be seen that nearly complete protein removal could be obtained with chloroform-octanol extractions. A constant DNA/RNA ratio of 2 was always obtained after preliminary purification. Complete removal of RNA could be effected by short incubation with crystalline RNAase followed by alcohol precipitation.

Although chloroform-octanol extraction was most frequently employed for protein removal, we have employed other methods which are less tedious and in which losses of DNA are minimized. In table 4 are summarized some of the methods employed. Treatment with 0.1% sodium desoxycholate was found to be the most efficient method, particularly when performed twice. Its disadvantage is that the desoxycholate is ex-

TABLE 4. DEPROTEINIZING PROCEDURES FOR DNA (B. SUBTILIS 23)

Method	DNA/Protein
Original (2 M NaCl)	0.3
Chloroform-octanol (exhaustive)	>40
Sodium desoxycholate (0.1%) 1×	4.2
Sodium desoxycholate (0.1%) 2×	>50
XE-64 (acetate, pH 5.0)	1.8

tremely difficult to remove completely. The ion exchanger XE-64 was employed also, but with only moderate success. Its advantage, however, lies in its ability to remove associated compounds such as certain polysaccharides which cannot be removed by the other methods.

The isolation procedure described in table 3 results in DNA solutions which are biologically active. Protein removal usually increases the activity, possibly by providing smaller molecules which can more readily penetrate cell wall barriers. Biological activity could be maintained optimally if the DNA was dissolved in 2 M sodium chloride solution. Lower ionic strength often led to inactivation. We are unable to explain this finding at the present time, except to suggest that high ionic strength may prevent irreversible binding of DNA by other components such as polysaccharides or protein. Otherwise the deproteinized DNA solutions are extremely stable and can be held at 4°C for longer than a year without any detectable loss of activity. For most of the experiments we employed protein-free DNA still containing the RNA moiety, since in certain experiments we have obtained higher activities in such a mixture.

Figure 1 shows a typical dose-response curve with the indole-requiring strain 168. It can be seen that a linear relation exists between the log of the quantity of DNA from less than 10^{-5} to approximately 10^{-1} γ of DNA and the log of the number of converted organisms. The curve then shows saturation. The number of converted organisms was obtained by counting colonies formed after 24 hours incubation on minimal agar lacking indole, after contact with DNA for 90 minutes at 37°C followed by treatment with deoxyribonuclease (50 γ/ml) for 10 minutes. The total number of recipient cells was determined by plating on minimal agar to which indole or L-tryptophan is added. In this experiment approximately 1% of the culture was transformed at saturation levels of DNA although higher frequencies have occasionally been observed. This type of data is highly reproducible, so long

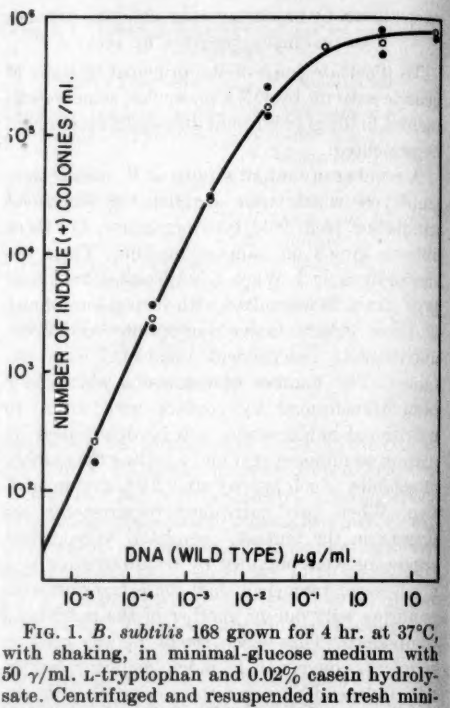


FIG. 1. *B. subtilis* 168 grown for 4 hr. at 37°C, with shaking, in minimal-glucose medium with 50 γ/ml. L-tryptophan and 0.02% casein hydrolysate. Centrifuged and resuspended in fresh minimal-glucose medium with 5 γ/ml L-tryptophan and 0.01% casein hydrolysate. DNA at various concentrations added and incubated at 37°C for 90 min. with shaking, followed by DNAase (50 γ/ml) for 10 min. Plated on glucose-minimal agar and incubated 24 hr. at 37°C. The number of transformants is determined from the number of colonies on minimal agar. The total number of recipient cells is determined from the number of colonies on minimal agar to which indole or L-tryptophan is added.

as the cells are in the proper state of growth. In our initial experiments (12) we employed germinating spores which were difficult to reproduce precisely. Subsequently we found that vegetative cells, provided that they were growing under precise conditions of semi-starvation, were highly transformable in a reproducible fashion.

It was observed that cells grown optimally in rich medium, containing adequate amounts of amino acids, gave little or no transformation. Table 5 presents a brief summary of one of the important conditions required for transformation in strain 168.

It is seen that when these cells are first grown in a glucose-minimal medium containing L-tryptophan (50 γ/ml) or yeast extract for 4 hours

at 37°C, then washed and resuspended in glucose-minimal medium containing DNA isolated from a wild type organism, some transformation would occur in 60 minutes at 37°C. However, much greater transformation would occur if .01% yeast extract was employed in minimal medium. We subsequently found that L-tryptophan or indole (5 γ/ml or less) would replace the yeast extract. Now if higher concentrations of yeast extract, or if a moderately high concentration of amino acids was added to the cells prior to DNA addition, much less transformation would result. It is of some interest in this connection that transformation of capsular and antibiotic resistance characters in Pneumococci requires the presence of serum albumen and Ca^{++} ions (10^{-3}–10^{-4} M). We had observed that high concentrations of serum albumen in the absence of L-tryptophan would in fact promote transformation of indole biosynthesis in *B. subtilis*, but this is probably due to the presence of adsorbed L-tryptophan.

In addition to L-tryptophan we find that magnesium ions and a low concentration of casein hydrolysate (.01%) are required for optimal results. Recent experiments (Anagnostopoulos and Spizizen, unpublished results) have shown that the casein hydrolysate could be replaced by L-histidine and further that L-histidine could be replaced by other chelating compounds which have a high affinity for cupric ions. Evidently the requirement for casein hydrolysate is due to the necessity for chelating cupric or related ions.

In figure 2 are presented growth curves of the indole-requiring *B. subtilis* strain 168 grown under optimal conditions for transformation. The culture was grown at 37°C in a minimal medium containing 50 γ/ml of L-tryptophan and .02% casein hydrolysate for 4 hours, centrifuged and resuspended in 10 volumes of minimal medium containing 5 γ/ml tryptophan and .01% casein hydrolysate at 37°C. Using 10 minute contact periods with DNA followed by DNAase addition, we determined that approximately 1½ hours after the initiation of the second growth phase the cells could be transformed at maximal efficiency. We can say that the cells are highly 'competent' at this particular stage of growth. We found that 10 minutes or less was all that was required for maximal transformation to indole-independence at this time and in addition, the cells at this period of time could be washed and transformed with DNA without the addition of any other factors.

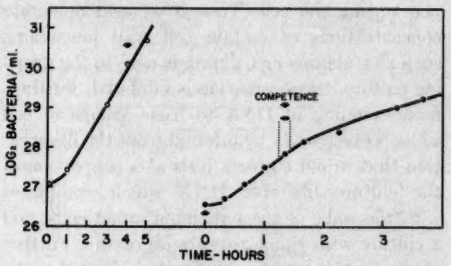


FIG. 2. Growth curves of *B. subtilis*, strain 168 in glucose-minimal medium containing L-tryptophan and casein hydrolysate under conditions employed for transformation studies. The first curve depicts growth at 37°C in glucose-minimal medium containing 50 γ/ml L-tryptophan and .02% casein hydrolysate. The arrow indicates that at 4 hr. the cells were centrifuged and resuspended in 10 volumes of fresh glucose-minimal medium containing 5 γ L-tryptophan per ml and .01% casein hydrolysate. The second curve describes the subsequent growth at 37°C. At 1½ hr. after the initiation of the second growth phase, maximal efficiency for transformation to indole-independence could be demonstrated. This is the so-called 'competence' time.

TABLE 5. REQUIREMENTS FOR B. SUBTILIS 168 (INDOLE-) TRANSFORMATION

DNA (wild type)	Number of wild type colonies formed in 60 min.		
	Minimal Medium	Minimal + .01% Yeast Extract	Minimal + 5 γ L-tryptophan/ml
γ/ml	×10⁵	×10⁵	×10⁵
20	5.9	10	20
2	0.64	7.1	12
0.2	0.5	4.2	5.0

B. subtilis 168 grown for 4 hr. at 37°C in glucose-minimal + 50 γ L-tryptophan/ml + .02% casein hydrolysate and washed prior to addition of DNA and fresh glucose-minimal medium.

We can only speculate as to the nature of the 'competence' period. Fox and Hotchkiss (16) and other workers have shown that in Pneumococci, fixation of P^{32} labeled DNA would occur in the absence of growth but amino acids and glucose in addition to serum albumen and calcium were necessary for the fixation. They suggest a specific site of binding of DNA. Our experiments show similar features except that no factors appear to be required once competence is achieved. Our experiments suggest further that cell walls as synthesized under optimal growth conditions may bind DNA irreversibly and prevent it from

penetrating the cell. Thus if we add moderate concentrations of certain cell wall precursors, such as L-alanine or L-glutamic acid to the growing culture, transformation is inhibited. Furthermore, binding of DNA to basic groups of cell walls probably can be inferred from the observation that in old cultures lysis of a proportion of the culture liberates DNA which complexes with the walls of the remaining intact cells, and a culture with slimy appearance occurs. Further studies in this area are obviously indicated.

EFFECT OF DNA-ASE AND INHIBITORS

One of the criteria which is used to establish the genetic activity of DNA in transforming systems is the specific inactivation by deoxyribonuclease. Complete inactivation of *B. subtilis* DNA transformation can be effected with 0.5 γ/ml or less of crystalline deoxyribonuclease in the presence of magnesium ions. When the enzyme was added at different times during a 90-minute incubation of a transformation mixture containing cells previously grown for 4 hours and transforming DNA, the following observations were made, (table 6). It can be seen that deoxyribonuclease inactivated transforming activity even when added at 40 minutes, indicating that the wild type DNA did not completely enter the cell until close to the critical competence time. When DNA isolated from a different type of organism, *E. coli*, was added to a transformation mixture, inhibition of transformation occurred. Even after 60 minutes, *E. coli* DNA was found to inhibit considerably, indicating that competition for the genetic site would occur after the DNA had combined with or entered the cell.

Another observation of interest is the finding that DNA of the homologous type, i.e. isolated from the organism to be transformed, would also compete with DNA from the wild type, but not as effectively as the heterologous DNA from *E. coli*. Homologous DNA did not by itself have any detectable genetic activity on the cells of strain 168.

TABLE 6. KINETICS OF DNA-ASE AND INHIBITOR ACTIVITIES

(*B. subtilis* 168 (indole-) recipient)

Additions*	Number of indole $^+$ converted/ml $\times 10^4$ (90 min. 37°C) (Time of addition)				
	0 min.	10 min.	30 min.	40 min.	60 min.
None	16				
50 γ/ml DNAase	0	0.2	0.2	1.6	11
40 γ/ml *E. coli* B-4, DNA	0.02	0.1	0.3	0.9	3.4
20 γ/ml *B. subtilis* 168 DNA	1.1	1.3			

* Wild type DNA, 0.2 γ/ml, added to *B. subtilis* 168, grown for 4 hr. at 37°C in glucose-minimal medium with 50 γ/ml L-tryptophan and .02% casein hydrolysate, centrifuged and resuspended in fresh glucose-minimal medium with 5 γ/ml L-tryptophan and .01% casein hydrolysate. This was followed by incubation for 90 min. at 37°C with shaking, with or without additions at stated times. After 90 min., aliquots were plated for colony counts on glucose-minimal agar. After 24 hrs. incubation, the number of colonies which appeared were counted and this gives the number of indole $^+$ converted organisms.

GENETIC ACTIVITY OF DNA IN THE FORMATION OF SPECIFIC ENZYMES

In order to investigate the mechanism of the genetic activity of DNA, it is necessary to look at the enzymatic consequences of the DNA activity. The unique structures of the active centers of the enzymes may ultimately be a reflection of the unique configuration of the DNA. For example, the DNA transformations in Pneumococci cause the formation of an entirely new biosynthetic pathway for the synthesis of the specific capsular polysaccharides. Austrian and his co-workers (17) have brilliantly demonstrated that in the transformation of the non-capsulated Type III to capsulated Type I a minimum of three new biochemical systems are formed: *1*) oxidation of uridine diphosphoglucose to uridine-diphosphoglucuronic acid, *2*) epimerization of uridine diphosphoglucuronic acid to uridine-diphosphogalacturonic acid, *3*) polymerization of Type I capsular polysaccharide. Such complex biosynthetic components suggest multiple gene involvement and may account for the low frequencies of DNA conversions observed.

Enzymatic alteration by transforming DNA was also shown by Ephrussi-Taylor (18), again using DNA isolated from Type III pneumococci of the so-called LC or large-colony type. Not only were Type II unencapsulated pneumococci converted to Type III encapsulated by this DNA but also LC (or large-colony) forms were obtained. The LC character was converted independently of the capsular change, except for a small percentage of double type changes. The LC pneumococci were found to have lost their ability to oxidize lactate and in addition had a decreased O_2 consumption on glucose.

Viral T2 infection of *E. coli* B via DNA injection as demonstrated by the classical experiments of Hershey and Chase (19) also leads to the formation of new enzymes. Flaks and Cohen (20) first demonstrated that a new enzyme is formed following T2-DNA injection which converts deoxycytidylic acid to hydroxy-methylcytidylic acid. A whole array of new enzymes concerned with T2 DNA synthesis are apparently formed and details of their detection and isolation were reported at these meetings (21–23). In this case the unique structure of the genetic DNA containing an unusual pyrimidine base is apparently responsible for the formation of a number of enzymes involved in its biosynthesis.

A number of inducible enzyme systems have been formed by DNA transformation (table 7). Organisms which are not able to form this type of enzyme can be altered by DNA transformation so that they become potentially capable of doing so in the presence of the inducer. The inducible enzyme mannitol phosphate dehydrogenase, a DPN-linked enzyme which oxidizes mannitol phosphate to fructose-6-phosphate, has been found by Marmur and Hotchkiss (13) to be formed in Pneumococci by DNA transformation. It makes no difference whether the DNA is isolated from an adapted strain or from a non-adapted strain which has the potential for making enzyme.

We have made mutants by ultraviolet irradiation of the indole-requiring *B. subtilis*, strain 168, which are unable to grow adequately on lactose or sucrose as the carbon source and produce tiny colonies on minimal agar containing L-tryptophan and the carbon sources respectively. The mutants were found to contain very low amounts of β-galactosidase and sucrase and the amounts of these enzymes could not be increased by induction. The enzymes in the wild type are inducible, but not in the mutants. By using colony size as an indicator of enzyme potentialities in the sucrose and lactose mutants, we have examined the genetic activity of DNA from wild type organisms. We have found that conversion to large-type colonies on sucrose-minimal agar containing L-tryptophan occurred with approximately the same frequency as conversion to indole-independence by colony counts on glucose-minimal agar (table 8). However, lactose conversion was not quite as efficient. When transformed cultures were plated on minimal medium lacking indole but containing sucrose as the only carbon source, a smaller number of large-type colonies could be detected. Approximately 3.5% of the single-character conversions were double transformations, which indicates some association or linkage of these characters. Almost all lactose-positive organisms are sucrose-positive, indicating very close linkage in these two genes.

The large colonies on sucrose medium (obtained by transformation) were individually transferred to a rich nutrient medium, and subsequently induced by sucrose. Lysozyme lysates of such induced organisms were then tested for sucrase hydrolytic activity (table 9). The original sucrase noninducible organism had approximately 1/10 the amount of enzyme in terms of specific activity as compared with the transformants—an amount which was present in sucrase-inducible cells prior to induction. The sucrase activity of the transformants was somewhat higher than that in wild type organisms from which the genes were isolated. This appears to be reproducible and we are unable to explain it.

In order to determine if the recipient organism may not be capable of hydrolysing sucrose because of some inhibitor, we mixed extracts of donor (sucrase-inducible) and recipient (sucrase-noninducible) prepared as previously mentioned. As seen in table 9, no inhibition was observed, so that we may conclude that in the transformed organism, potential ability to form enzyme was

TABLE 7. GENETIC ACTIVITY OF DNA IN FORMATION OF SPECIFIC ENZYMES

Enzyme System	Microorganism
Mannitol phosphate dehydrogenase	*D. pneumoniae* (13)
Salicin utilization	*D. pneumoniae* (15)
β-galactosidase	*B. subtilis**
Sucrase	*B. subtilis**

* Spizizen, unpublished results.

TABLE 8. SUCRASE AND β-GALACTOSIDASE CONVERSION

(Recipient: Lac- Suc- Ind- *B. subtilis* at 1.2×10^8/ml after 60 min.)

Wild type DNA	Indole+ converted, 60 min.	Sucrose+ converted, 60 min.	Lactose+ converted, 60 min.
γ/ml	$\times 10^5$/ml	$\times 10^5$/ml	$\times 10^5$/ml
20	8.6	8.3	4.2
2	7.2	5.9	1.0
2	double transformants (Indole+ Sucrose+) 0.25		

presumably effected by the transformation. We are continuing these studies by fractionating the proteins of the mutant and wild type organisms in order to determine if the enzyme protein or only the active site is involved.

In connection with these studies on sucrase conversion we have recently made an observation that may throw some light on the transformation process. We have employed DNA preparations which still contain the RNA moiety found to be associated with the DNA during our isolation procedure. When such preparations were treated with RNAase, we observed no effect on indole transformation in the sucrose-negative mutant of strain 168. However, sucrase-transformants were markedly reduced in number (table 10).

A possible explanation for this observation is that RNA carried in by the DNA is somehow involved in the formation of some sucrase enzyme, sufficient to permit survival on sucrose plates. It was shown by Monod (24) that lysis of *B. subtilis* will occur in the absence of a carbon source. Now there may be some time required for the sucrase-directing activity of the new gene to be manifest. During this period when the new biological system is being established, many organisms may lyse. The presence of RNA from the wild type organism may thus permit survival of a large proportion of the cells undergoing transformation.

TABLE 9. SUCRASE ACTIVITY OF EXTRACTS

Lysozyme Extracts of Organism after Sucrose Induction	Specific Activity of Extract (μM glucose*/mg protein)
Donor: *B. subtilis* wild (sucrose$^+$)	7.8
Recipient: *B. subtilis* 168, sucrose$^-$	1.1
Transformants (average of 10)	12.9
Donor + recipient (extracts)	10.0

* Glucose equivalents, which represent a mixture of glucose and fructose.

TABLE 10. EFFECT OF RIBONUCLEASE ON SUCRASE CONVERSION BY DNA

Wild type DNA	RNAase	Indole$^+$ converted, 90 min.	Sucrose$^+$ converted, 90 min.	% Reduction in sucrose$^+$ conversion by RNAase
γ/ml	γ/ml	×10^6/ml	×10^6/ml	
20	0	6.0	3.7	
2	0	3.8	2.0	
20	20	6.0	1.0	73
2	20	3.0	0.24	88

Total sucrose-indole-6 × 10^7/ml.

SUMMARY AND CONCLUSIONS

Direct demonstration of the genetic activity of deoxyribonucleic acid has been obtained in the studies of transformation in microbial systems. The following summarizes the principal features of such systems, as exemplified by the new *B. subtilis* system:

1) The genetic activity is always associated with the DNA fractions during purification and is inactivated by low concentrations of magnesium-activated DNAase.

2) A typical dose-response curve for single genetic markers is obtained. A straight-line relationship is obtained for the log of DNA concentration versus the log of the number of cells transformed over a wide range of DNA concentrations.

3) Inhibition of transformation by DNA isolated from unrelated organisms occurs. Some inhibition can also be demonstrated with relatively higher concentrations of DNA isolated from the recipient organism, that is, the one to be transformed. No genetic activity can be demonstrated by the competing DNA's when used by themselves.

4) Replication of the genetically-donated DNA can be demonstrated by the isolation of genetically active DNA from transformed organisms.

The new *Bacillus subtilis* transformation system has a number of advantageous features arising from the fact that these organisms can be cultivated on simple synthetic media. Mutants with nutritional requirements or other blocks in biochemical systems can be readily isolated. An unlimited number of enzyme 'markers' can thus be employed for biochemical investigations of the role of DNA in the control of synthesis in the cell. We have studied the transformation of inducible β-galactosidase and sucrase enzymes. Two important aspects of these transformations require investigation: *1*) Can the DNA be fractionated so that specific components can be isolated for each of these enzyme-controlling activities? *2*) What does the transformation achieve—is it the formation of whole new proteins or of active centers only?

REFERENCES

1. TATUM, E. L. AND J. LEDERBERG. *J. Bact.* 53: 673, 1947.
2. PARDEE, A. B., F. JACOB AND J. MONOD. *Compt. rend.* 246: 3125, 1958.
3. ZINDER, N. D. AND J. LEDERBERG. *J. Bact.* 64: 679, 1952.

4. AVERY, O. T., C. M. MACLEOD AND M. MCCARTY. *J. Exper. Med.* 79: 137, 1944.
5. MORGAN, T. H. *J. Exp. Zool.* 11: 365, 1911.
6. HOTCHKISS, R. D. In: *The Nucleic Acids*, edited by E. Chargaff and J. D. Davidson. New York: Acad. Press, 1955, vol. II, p. 446.
7. ALEXANDER, H. E. AND G. LEIDY. *J. Exper. Med.* 93: 345, 1951.
8. ALEXANDER, H. E. AND W. REDMAN. *J. Exper. Med.* 97: 797, 1953.
9. BOIVIN, A., A. DELAUNAY, R. VENDRELY AND Y. LEHOULT. *Experientia.* 1: 334, 1945.
10. HOTCHKISS, R. D. *Cold Spring Harbor Symposia Quant. Biol.* 16: 457, 1951.
11. ALEXANDER, H. E. AND G. LEIDY. *J. Exper. Med.* 97: 17, 1953.
12. SPIZIZEN, J. *Proc. Nat. Acad. Sc. U. S.* 44: 1072, 1958.
13. MARMUR, J. AND R. D. HOTCHKISS. *J. Biol. Chem.* 214: 383, 1955.
14. THOMAS, A. J. AND R. M. KLEIN. *Nature, London* 183: 113, 1959.
15. AUSTRIAN, R. AND M. S. COLOWICK. *Bull. Johns Hopkins Hosp.* 92: 375, 1953.
16. FOX, M. S. AND R. D. HOTCHKISS. *Nature, London.* 179: 1322, 1957.
17. AUSTRIAN, R., H. BERNHEIMER, E. E. B. SMITH AND G. T. MILLS. *Cold Spring Harbor Symposia Quant. Biol.* 23: 99, 1958.
18. EPHRUSSI-TAYLOR, H. *Exp. Cell Res.* 6: 94, 1954.
19. HERSHEY, A. D. AND M. CHASE. *J. Gen. Physiol.* 36: 39, 1952.
20. FLAKS, J. AND S. S. COHEN. *Biochim. et biophys. acta* 25: 667, 1957.
21. ZIMMERMAN, S. B., S. R. KORNBERG, J. JOSSE AND A. KORNBERG. *Fed. Proc.* 18: 359, 1959.
22. SOMERVILLE, R. AND G. R. GREENBERG. *Fed. Proc.* 18: 327, 1959.
23. KOERNER, J. F. AND M. S. SMITH. *Fed. Proc.* 18: 264, 1959.
24. MONOD, J. *Ann. Inst. Pasteur* 68: 444, 1942.

AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Topics of Current Interest in Biochemistry[1]

Chairman: SEVERO OCHOA

CHEMISTRY OF BACTERIAL CELL WALLS

FRIEDRICH ZILLIKEN

Biochemistry Department, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

REVIEWING THIS interesting topic it appears that a considerable amount of work has accumulated during the past 5 years particularly from studies of Drs. Salton (1), Cummins and Harris (2–4), McQuillen (5), Work (6), Weidel (7), Park and Strominger (8, 9) to name only a few laboratories which have been actively engaged in this field. It seems to be my purpose to summarize their contributions briefly and by the very nature of this presentation selectively, and furthermore to integrate their findings with our own work in this field.

It seems no longer necessary to apologize for the intrusion of biochemists into what used to be a purely bacteriological field. Needless to say the problem in its morphological and biofunctional aspect is of highest significance to all concerned with general activities of microorganism especially those involving the biosynthesis of mucoproteins, the action of antibodies, chemotherapeutic agents and the mechanism of transformation.

Bacterial cell walls provide the vital bacterial cytoplasm, covered only by the ultra thin membrane, with a form-giving framework varying in texture from fine to coarse, thus giving rigidity to the bacterium but nevertheless allowing for permeability of nutrients varying in size from ions to molecules of molecular weight of several millions.

Until recently the chemical nature of the cell walls was unknown, although speculative guesses as to their chitinous or mucoid nature were made. Technical developments in the past few years have resulted in the identification of many constituents of these walls and have shown the existence of a new type of molecule differing from polysaccharides and other known mucoproteins. A method of preparation of relatively homogenous walls from mechanically disrupted cells has been developed by Salton and his colleagues (10). Total separation of walls was confirmed by use of the electron microscope, the electron dense cytoplasm being removed by washing and differential centrifugation, leaving the cell walls as collapsed balloon-like structures (fig. 1). Such wall preparations are composed of lipids, carbohydrates, amino acids, hexosamines and phosphorous in varying quantities, but are devoid of any nucleic acids, purines or pyrimidines. The thickness of the bacterial wall varies from 10–20 mμ and comprises about 25% of the dry weight of bacteria hence its synthesis is a major function of the cell. The data compiled in figure 2 have been obtained from either chemical degradation of isolated cell walls or specific, enzymic attack by lysozyme or bacteriophage, although these studies revealed the walls to possess highly individualistic chemical characteristics some interesting similarities within different genera and within the gram-positive and -negative groups of bacteria have emerged. The walls of gram-positive bacteria are less complex than those of gram-negative microorganism particularly with respect to their amino acid content. Neither aromatic nor sulfur containing amino acids have been detected in the former. With slight variations similar compositions have been found for some 60 gram-positive bacteria, the major amino acids being D and L-alanine, D-glutamic acid, L-lysine and or α,ϵ-diaminopimelic acid in its LL, DD or meso form. There

[1] Atlantic City, New Jersey, April 16, 1959.

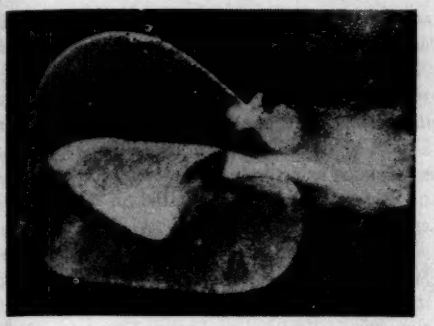


FIG. 1. Cell walls of *Bacillus megaterium* showing several adsorbed phage particles (×9,200). From Bacterial Anatomy, Cambridge, University Press, 1956.

are significant differences in the total lipid and aminosugar content in comparing the walls of gram-positive versus gram-negative organism, and in addition to D-glucosamine and D-galactosamine a novel, acidic amino sugar is present in both groups.

The elegant work of Weidel (7) with T-even phages has shown that walls of gram-negative organism contain in addition to a large lipoprotein layer also a lipopolysaccharide—believed to be the receptor for T-4 phage—very similar in structure to the basal unit of gram-positive bacteria.

As to the phosphorous content it is noteworthy that Baddiley (11) and associates have isolated polymers of ribitol-5′-phosphate from cell walls of 3 gram-positive bacteria—the so called teichoic acids, which seem to be α-glycosidically bound to either glucose or glucosamine, the latter being in ester linkage with alanine (fig. 3). Teichoic acid is apparently activated and transfered as a Cytidine diphosphonucleotide.

More insight into the fine structure of cell walls has emerged from 3 different and independent lines of investigations.

In 1954 Strange (12) observed that nondialysable mucopeptides could be obtained from walls and spores of 3 gram-positive Bacillus species by the action of a spore lytic enzyme. Although the composition varied slightly from one species to another these peptides exhibited both qualitatively and quantitatively a close similarity with the basal cell wall structure of gram-positive microorganism. These peptides were attacked by lysozyme and after hydrolysis found to be composed of alanine, glutamic acid, α,ϵ-diaminopimelic acid and an unidentified amino sugar named muramic acid. Cummins and Harris (2) have found this novel amino sugar in more than 60 gram-positive strains and Weidel (7) and we (E. Kean and F. Zilliken, MS in preparation) have indicated its presence also in cell walls of different *E. coli* strains. N-deacetylated muramic acid has been obtained in crystalline form by Strange (13) from 6N mineral acid hydrolysates of spore peptides and bacterial cell walls and he has proposed the tentative structure to be a 3-O-carboxyethyl-hexosamine (fig. 4.)

The structural proof rested essentially on the consumption of periodate reducing properties and the isolation of propionic acid after vigorous hydrolysis with hydroiodic acid in the presence of red phosphorous. The 3-O-substitution was derived from a characteristic Elson-Morgan reaction specific for 3-O-substituted hexosamines (14) giving an absorption maximum et λ max = 512 mμ. So far there has been no absolute evidence brought forward as to whether this is indeed the naturally occurring form of muramic acid in bacterial cell walls, particularly since the

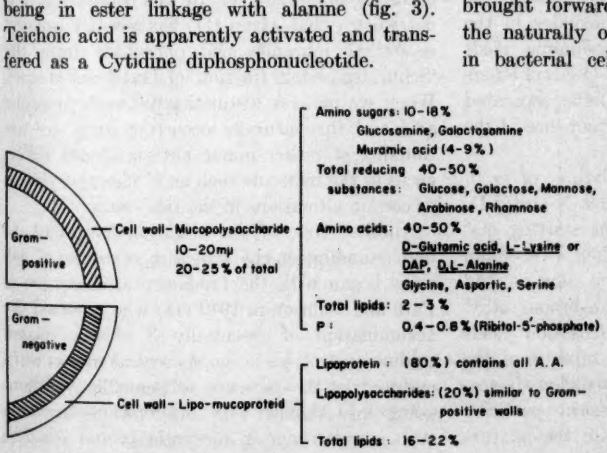


FIG. 2. Chemical composition of bacterial cell walls.

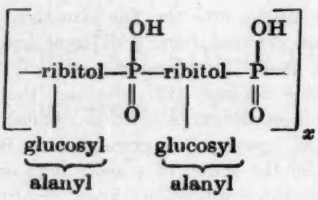


FIG. 3. Teichoic acid.

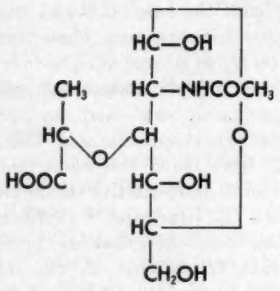


FIG. 4. N-acetyl-muramic acid.

product has been always obtained from rather drastic treatments, of either bacterial cell walls or their precursors, thus possibly destroying any fine structure present in the naturally occurring molecule. Most recently Strange (15) has reported the synthesis of 3-O-lactyl-D-glucosamine and a separation into its diastereoisomers with the result, that 3-O-D-lactyl-D-glucosamine possesses the same specific rotation as muramic acid obtained from 6 N acid hydrolysates of spore peptides from *B. megaterium* $[\alpha]_D^{25°}$ was given in both instances as +109°.

Since 3-O-lactyl ethers of N-acetyl hexosamines are of general interest as intermediates in the conversion to 2,6-anhydro-nonosaminic acids we have synthesized chemically 3-O-lactyl ethers of N-acetyl-D-glucosamine and have separated them into diastereoisomers. The sequence of the synthesis is outlined in figure 5.

α-Methyl-, the anomeric mixture of α,β methyl or β-Ethyl-4,6-benziliden-N-acetyl-D-glucosaminide have been used as starting material. These are converted into their 3-O-sodium salts with sodiumhydride in dry dioxane and without isolation reacted with α-bromo ethyl propionate. The assymetric condensation yields in 50–60% the diastereoisomeric mixture of the corresponding 3-O-lactyl-4,6-benzilidene-N-acetyl-D-glucosaminides. Since classical partition chromatography failed to separate the mixture we converted the latter into 3-O-D,L-lactyl-D-glucosamine by means of hydrolysis with 2.5 N hydrochloric acid for 4 hours. We then N-acetylated the mixture. Separation into D- and L-diastereoisomers was accomplished with ease on Dowex columns in the formate form employing a formic acid water gradient elution. The L-Diastereoisomer was isolated in form of its crystalline α-methyl-3-O-L-lactyl-N-acetyl-D-glucosa minide derivative ($[\alpha]_D^{25°}$ = +62.5°). The latter sublimes at 175°C. under conversion into its Lactone $[\alpha]_D$ = +127° (extrapolated to 0 time) → +66.0° (equilibrium rotation at 36 hr.). Acid hydrolysis of N-acetyl muramic acids under conditions generally employed for the hydrolysis of bacterial cell walls gives rise to at least two other products namely, 3-O-ethoxy-glucosamine and a lactame of the acid. This might be noteworthy for those who have been concerned with the identification of disaccharides resulting from lysozyme action on isolated bacterial cell walls (16). Furthermore we observed, that synthetically prepared 3-O-D-lactyl-D-glucosamine ($C_9H_{17}O_7N$ (251.3), M.p. 155° (under decomp.) $[\alpha]_D^{25°}$ + 165.0° (extrapolated to 0 time) → +123.0° (equilibrium rotation at 3 hr. c = 3.5; H_2O) is identical with muramic acid as isolated after acid hydrolysis of either bacterial cell walls or lysozyme digests thereof. Besides a slight deviation in optical rotation this is consistent with the finding of Strange (15).

There is now a general agreement that the novel amino sugar first isolated by Strange (12) is indeed a 3-O-D-lactyl-D-glucosamine. No conclusion may be derived from this observation as to the true chemical structure of the naturally occurring muramic acid. In this connection it is noteworthy that Agren (17) has recently isolated crystalline muramic acid phosphate from the 'Schneider protein fraction' of *Lactobacillus casei*. When we make a distinction between muramic acid and the naturally occurring form, we are thinking of rather minor but significant differences in the molecule such as 6′-phosphorylation or certain alterations in the side chain.

Another line of investigation pertinent to understanding of the structure of bacterial cell walls began with the fundamental discovery of Park and Johnson in 1949 (18) who reported the accumulation of essentially 3 closely related uridine nucleotides in *Staphylococcus aureus* when cultured in the presence of penicillin. Without going into details with observations covering such a wide scope in biosynthesis and selective

inhibitions of bacterial cell wall synthesis, Park and Strominger (8, 9), in a number of admirable contributions have presented a tentative structure of what might be considered the repeating unit of an activated cell wall precursor (fig. 6). The ratio of Muramic acid:alanine: glutamic acid:lysine = 1:3:1:1, a ratio found also in lysozyme digests of whole bacteria and phage treated *E. coli*. Noteworthy is the absence of glucosamine and glycine in the presented scheme. Support of the amino acid sequence has been most recently presented by Strominger (20).

Similar valuable information toward the fine structure of bacterial cell walls begin to emerge

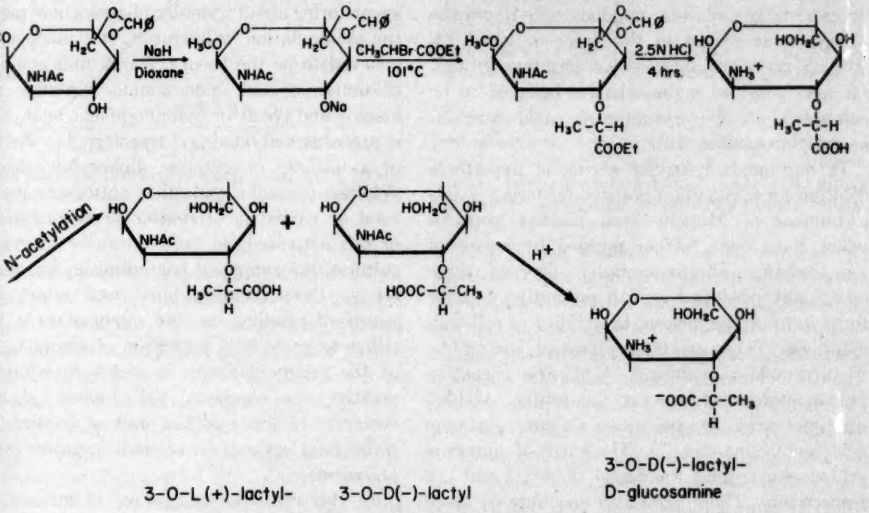


FIG. 5. Chemical synthesis of muramic acids.

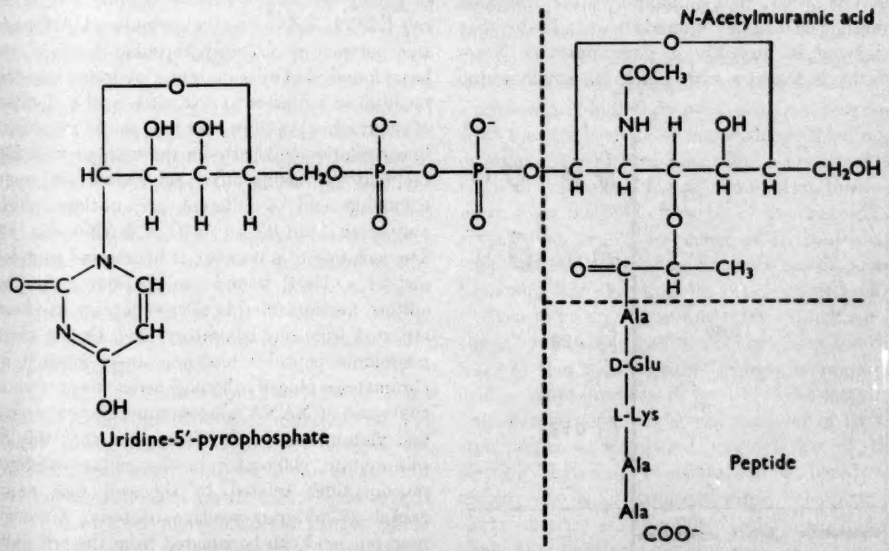


FIG. 6. A tentative structure of the principal nucleotide that accumulates in penicillin-treated *Staphylococcus aureus*. The sequence of amino acids is one of several possible arrangements (Strominger, 1955).

from the action of lysozyme on lysozyme-sensitive whole microorganism and isolated cell walls. Karl Meyer was the first to extract the lysozyme substrate in a soluble form, demonstrating the release of reducing substances during its action (21). Salton has recently critically reviewed the properties of lysozyme (22). He has shown that the enzyme when acting on susceptible cell walls liberates large molecular nondialysable fragments of molecular weight in the order of 10–15,000 with N-acetylhexosamines as terminal groups. He also obtained a disaccharide believed to be composed of N-acetyl-muramic acid and N-acetyl-glucosamine (16).

In our hands lysozyme acting in hypertonic solution on whole gram-positive bacteria releases a number of Morgan-Elson positive peptides which have been further purified by means of ion exchange chromatography. One of these specifically possessed growth promoting activity for a *lactobacillus* lacking the ability of cell wall synthesis. These peptides represent dialysable, weakly acidic, reducing, ninhydrin negative mucopeptides which on hydrolysis yielded muramic acid, glucosamine, alanine, glutamic acid and lysine (fig. 7). The ratio of muramic acid to glucosamine was found to be 1:1 and 1:2 respectively. There were also encountered trace amounts of glycine and aspartic acid. End-group analysis with potassiumborohydride revealed muramic acid to be terminal which indicates that lysozyme is probably a muraminidase. Noteworthy is the ease with which the acidic amino sugar can be released from the mucopeptides favoring the presence of amino acid ester bonds in Baddiley's (11) interpretation rather than the occurrence of true peptide bonds linking the acidic amino sugar with the peptide moiety.

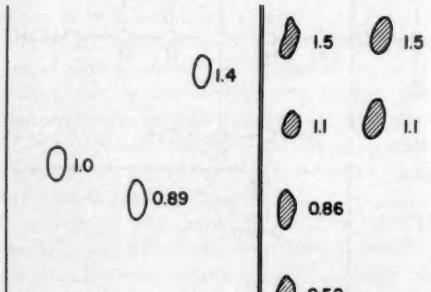


FIG. 7. Paperchromatogram of dialysable Lysozyme digests of Micrococcus lysodeicticus.

Summarizing the effect of certain antibiotics, bacteriophages and lysozyme on bacterial cell walls, one is tempted to think that all three agents bring about a similar phenomenon, namely the accumulation or liberation of a mucopeptide believed to be the basal cell wall unit composed of muramic acid, glucosamine, glutamic acid, alanine and lysine or diaminopimelic acid, which is probably activated and transferred in the form of a uridine- or cytidine diphosphonucleotide. Whereas penicillin and other antibiotics prevent total or partial incorporation or polymerisation of this activated cell wall precursor in growing cultures, lysozyme and bacteriophage are able to release this repeating unit from intact walls which, depending on the circumstances leads either to protoplast formation of complete lysis of the microorganism. It seems therefore imperative to elucidate the precise chemical structure of this essential unit of bacterial cell walls from gram-positive and -negative microorganisms.

In this context it might be of interest that Goebel and Barry (23) reported the occurrence of a polymer of N-acetyl-neuraminic acid in *E. coli* K235 L + O named colominic acid. Although this polymer is probably capsular in origin, we have found that this mutant elaborates into the medium in addition to colominic acid a number of neuramino peptides. There is found an almost linear relationship between the extinction at 260 mμ and the neuraminic acid content of crude colominic acid of different preparations which may vary from 12 to 57% of NANA (fig. 8). The isolation of a number of neuramino peptides and of a UDP bound mucopeptide from the culture medium of this microorganism has been reported from our laboratory (24). One of these neuroaminopeptides has now been isolated in chromatographically homogeneous form and is composed of NANA, glucosamine, alanine, lysine and glutamic acid (25). It appears that this *E. coli* mutant elaborates in the culture medium mucopeptides related to the cell wall muco complexes of gram-positive bacteria. Although muramic acid can be isolated from the cell walls of this mutant, no trace of the latter could be detected in these mucopeptides.

Our most recent studies have been concerned

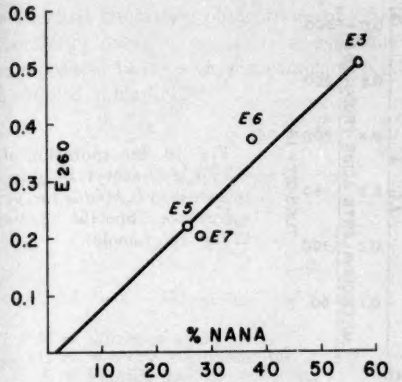


FIG. 8. Relationship of extinction at λ 260mμ of 0.02% solution of colominic acid and percent N-acetyl-neuraminic acid.

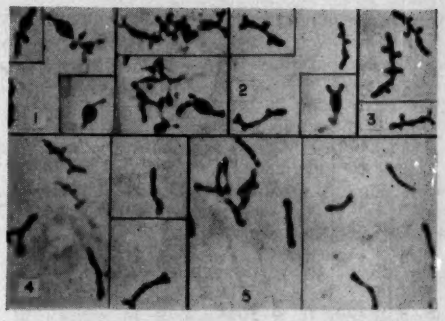


FIG. 9. Forty-hour cultures of *L. bifidus var. pennsylvanicus:* 1 = no growth factor present; 2 = 10 μg; 3 = 20 μg; 4 = 40 μg; 5 = 80 μg present. Growth factor may be β-Ethyl-N-acetyl-D-glucosaminide; N-acetyl-lactosamine or human milk.

with a gram-positive microorganism which seems promising in solving quite a number of problems pertinent to the biosynthesis of bacterial cell wall precursors particularly with respect to the nature of amino sugars involved. This bacterium is *Lactobacillus var. pennsylvanicus*, originally discovered by Paul György (26).

This mutant exhibits a specific growth requirement for β-glycosides of N-acetyl-D-glucosamine or N-acetyl-lactosamine (27, 28). This apparent discrepancy over different chemical structures possessing equal growth promoting activity as well as the absolute amounts required for optimal growth suggested that both substances might be converted to a common intermediate engaged in a major chemical reaction of the cell. It seemed possible that the growth factors might be required for the formation of the bacterial cell wall of this microorganism. This viewpoint was supported by the fact that lysozyme digests of other gram-positive microorganism were found to possess growth promoting activity and by the formation of atypical forms in the absence of β-Alkyl-N-acetyl-D-glucosaminides, N-acetyl-lactosamine or human milk, as shown in figure 9 (29).

The organism varies from the bulbous forms with no aminosugar through knobbed forms and finally to simple rods completely lacking the bifid structure when cultured in the presence of high concentrations of growth factor. Encouraged by these striking morphological changes which resemble those of penicillin-treated cultures, we prepared [1-C^{14}] labeled α,β-methyl-N-acetyl-D-glucosaminide. The anomeric mixture consists of 15% of the β-anomer. The specific activity was 21,000 cpm/μmol. The rate of incorporation in relation to total acid production, turbidity, and cpm/mg of cells is shown in figure 10. Uptake of label proceeds rapidly during the log phase and reaches a maximum at approximately 15 hours. Striking morphological changes coincide exactly with the availability of the β-methyl-N-acetyl-D-glucosaminide. In the early growth phase up to the maximal incorporation we observed straight rods. In the later phase budding, and after the available glycoside has been extensively utilized, branching occurs (bifid forms) and finally the organism assumes swollen, bizarre forms as is shown in figure 11. Thus, we have been able to demonstrate the formation of bizarre forms in *Lactobacillus bifidus var. pennsylvanicus*, resulting from the deficiency of an amino sugar which is an essential growth requirement and a precursor for cell wall synthesis of this microorganism. Furthermore, even the bifid character of the organism is revealed to be a consequence of an inadequate supply of this same factor, since we can eliminate branching by elevating its concentration (30).

Even more gratifying was the identification of the newly incorporated component from the cell walls of this microorganism in form of muramic acid or more precisely in form of 3-O-D-lactyl-D-glucosamine possessing specific activity of 19,000 cpm/μmole as compared with 22,000 of the starting material. Identification is based on mobility on paper chromatograms, behaviour in electrophoresis at different pH and quantitative Elson-Morgan reaction with λ max. = 512 mμ as compared with crystalline, synthically obtained 3-O-D-lactyl-D-glucosamine (31).

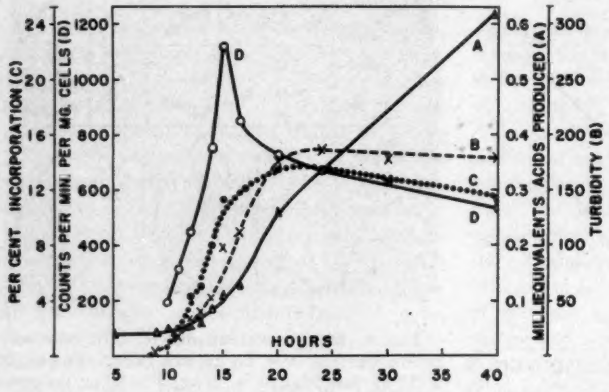


FIG. 10. Incorporation of [1-C^{14}]-α, β-N-acetyl-D-glucosaminide into *L. bifidus var. pennsylvanicus*. Specific activity 22,000 cpm/μmole.

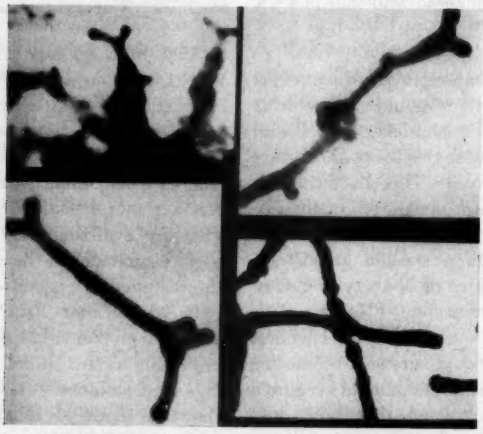


FIG. 11. Electron micrographs of *L. bifidus var. pennsylvanicus*. Upper left: without growth factor; upper right: with 10 μg; lower left: with 20 μg and lower right: with 40 μg of β-Ethyl-N-acetyl-D-glucosaminide present.

Our findings demonstrate that N-acetyl-D-glucosamine is a direct precursor in the biosynthesis of muramic acid, or more precisely of 3-O-D-lactyl-D-glucosamine. However no conclusion can be derived from this observation as to the true chemical structure of the naturally occurring form, particularly since synthetically prepared 3-O-D- or 3-O-L-N-acetyl-D-glucosamine did not exhibit any growth promoting activity for this microorganism. It is also noteworthy that N-acetyl-L-glucosamine, N-acetyl-D-galactosamine and N-acetyl-D-mannosamine are completely devoid of any growth promoting activity thus providing additional, biological evidence for the 2-deoxy-2-amino-D-glucopyranose configuration of muramic acid. The biosynthesis of muramic acid proceeds at the level of phosphorylated aminosugar intermediates and is at present under investigation employing a cell-free enzyme system. In this connection it is noteworthy that Strominger (32) recently indicated a pyruvic acid transfer from phosphoenolpyruvate to UDPAG with extracts of *S. aureus, E. coli* and aerobacter aerogenes.

As significant as the biosynthesis of muramic acid might be for the general problem of bacterial cell wall formation and the chemical synthesis of antimetabolites, our findings seem to provide an answer to the mode of action of the Bifidus factors. All factors isolated from natural sources or synthesized so far seems to be no more than precursors whose only function is to provide N-acetyl-D-glucosamine in a properly activated form in order to restore impaired cell wall synthesis of this microorganism.

Summarizing the information available at

present and integrating observations of different laboratories one may picture the cell walls of gram-positive bacteria in a preliminary fashion as outlined in figure 12.

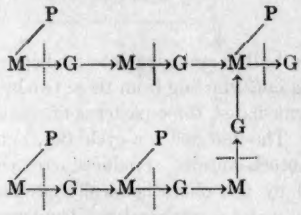


FIG. 12

The backbone of these strands is made of N-acetyl-D-glucosamine and Muramic acid, the latter carrying the peptide unit. Branching points to the next tidely packed chain occurs from muramic acid. Penicillin prevents the incorporation of M—P units (8, 9), Cycloserine the incorporation of terminal D-alanine units into the peptide moiety (33). Lysozyme and phage bring about liberation of G→M—P from straight chains, or G→M—P, and G→M from branching

G↑

points. Inner metal complexes between lysine and glutamic acid may increase rigidity and may contribute to an understanding of the versene effect with Lysozyme (34). O-acetyl groups, some of which may actually be present in form of O-alanyl groups, are in ester linkage with muramic acid. Their removal may contribute to lysozyme susceptibility via removal of steric hindrance for the enzyme (35).

In the preceding paragraph I have attempted to enumerate but a few experimental data which must be integrated into a still more comprehensive picture of the actual structure of bacterial cell walls.

I wish to express my gratitude to Dr. R. Lambert for the synthetical part of this work, to Dr. M. C. Glick and Mr. P. O'Brien for the microbiological and radioactive studies and to Mr. E. Kean for the purification of NANA-peptides. I am also indebted to Drs. T. Sall and S. Mudd, Microbiology Department, for electron microscopy and many stimulating discussions.

REFERENCES

1. SALTON, M. R. J. In: *Bacterial Anatomy*. London: Cambridge Univ. Press, 1956, p. 81.
2. CUMMINS, C. S. AND H. HARRIS. *Biochem. J.* 57: 32, 1954.
3. CUMMINS, C. S. AND H. HARRIS. *J. Gen. Microbiol.* 14: 583, 1956.
4. CUMMINS, C. S. *Intern. rev. Cytol.* 5: 25, 1956.
5. MCQUILLEN, K. In: *Bacterial Anatomy*, London: Cambridge Univ. Press, 1956.
6. WORK, E. *Nature, London* 179: 841, 1957.
7. WEIDEL, W. AND J. PRIMOSIGH. *Z. Naturforsch.* 12: 441, 1957.
8. PARK, J. T. AND J. L. STROMINGER. *Science*, 125: 99, 1957.
9. STROMINGER, J. L. *J. Biol. Chem.* 234: 1520, 1959.
10. SALTON, M. R. J. AND R. W. HORNE. *Biochim. et biophys. acta* 7: 177, 1951.
11. ARMSTRONG, J. J., J. BADDILEY, J. B. BUCHANAN, B. CARSS AND G. R. GREENBERG. *J. Chem. Soc.* 4344, 1958.
12. STRANGE, R. E. AND J. F. POWELL. *Biochem. J.* 58: 80, 1954.
13. STRANGE, R. E. AND F. A. DARK. *Nature, London* 177: 186, 1956.
14. CIFONELLI, J. A. AND A. DORFMAN. *J. Biol. Chem.* 231: 11, 1958.
15. STRANGE, R. E. AND L. H. KENT. *Biochem. J.* 71: 333, 1959.
16. SALTON, M. R. J. *Biochim. et biophys. acta* 22: 495, 1956.
17. AGREEN, G. AND C. H. DE VERDIER. *Acta Chem. Scand.* 12: 1927, 1958.
18. PARK, J. T. AND N. J. JOHNSON. *J. Biol. Chem.* 179: 585, 1949.
19. PARK, J. T. *J. Biol. Chem.* 194: 877, 885, 897, 1952.
20. STROMINGER, J. L. *Fed. Proc.* 18: 334, 1959.
21. MEYER, K., J. W. PALMER, R. THOMPSON AND D. HORAZO. *J. Biol. Chem.* 113: 479, 1936.
22. SALTON, M. R. J. *Bact. Rev.* 21: 82, 1957.
23. BARRY, G. T. AND W. F. GOEBEL. *Nature, London* 179: 206, 1957.
24. O'BRIEN, P. J. AND F. ZILLIKEN. *Biochim. et biophys. acta* 31: 543, 1959.
25. KEAN, E. Dissertation. In press.
26. GYÖRGY, P. *Pediatrics* 11: 98, 1953.
27. ZILLIKEN, F., C. S. ROSE, G. A. BRAUN AND P. GYÖRGY. *Arch. Biochem. and Biophys.* 54: 392, 1955.
28. TOMARELLI, R. M., J. B. HASSINEN AND F. W. BERNHARDT. *Arch. Biochem. and Biophys.* 48: 225, 1954.
29. GLICK, M. C., T. SALL, F. ZILLIKEN AND S. MUDD. *Biochim. et biophys. acta.* In press.
30. O'BRIEN, P., M. C. GLICK AND F. ZILLIKEN. *Biochim. et biophys. acta.* In press.
31. LAMBERT, R. AND F. ZILLIKEN. *J. Am. Chem. Soc.* In press.
32. STROMINGER, J. L. *Biochim. et biophys. acta* 30: 645, 1958.
33. STROMINGER, J. L., H. R. THRENN AND S. S. SCOTT. *J. Am. Chem. Soc.* 81: 3803, 1959.
34. REPASKE, R. *Biochim. et biophys. acta* 22: 189, 1958.
35. ABRAMS, A. *J. Biol. Chem.* 230: 949, 1958.

PHOTOSYNTHETIC PHOSPHORYLATION[1, 2, 3]

ANDRE T. JAGENDORF

McCollum-Pratt Institute and Biology Department, Johns Hopkins University, Baltimore, Maryland

FORMATION OF ATP from light energy was postulated to be a part of the mechanism of photosynthesis as early as 1941 by Lipmann (1) Ruben (2) Emerson *et al.* (3) and others. Subsequently, evidence for light dependent phosphorylation by whole cells was obtained in a number of laboratories (4–8). (See (9) for a review of the whole cell experiments.) A direct demonstration of ATP formation by isolated cell-free particles was provided almost simultaneously by Arnon, Whatley and Allen with spinach chloroplasts (10) and by Frenkel with particles from *Rhodospirillum rubrum*, a facultative anaerobic photosynthetic bacterium (11). Since then ATP formation has been found in extracts of a number of other photosynthetic organisms, including *Chromatium* (12) and *Chlorobium*, (13) obligately anaerobic bacteria; *Spirogyra* (14), and a blue-green alga (15). Although not all groups in the plant kingdom have been examined yet, it now seems quite likely that phosphorylation will be an integral part of any complete photosynthetic system.

Any evidence so far available suggests that phosphorylation here, as in mitochondrial oxidative phosphorylation, depends basically on the functioning of an electron transport mechanism. The first things to be considered, then, are the current concepts on the nature of the electron transport pathways that are the basis for phosphorylation here. These concepts are in the form of reasonable suggestions for the broad outlines of the reactions; virtually nothing is known of the nature of the electron carriers in any detail yet.

The basic assumption is that the action of light produces both a reducing intermediate, and an oxidizing one. Starting from these two hypothetical intermediates, three patterns of reactions are possible. The first one is a cycle (fig. 1) in which the photochemically produced reductant is oxidized by the photochemically produced oxidant and water is regenerated. (The figure shows a water molecule as the source of both oxidant and the reductant; however it could also be some other molecule that is split as a result of the primary photochemical act). In bacteria the cycle may be completed through enzymes endogenous in the isolated chromatophores (16). Lucile Smith and Margareta Baltcheffsky for instance have obtained evidence that (17) in *Rhodospirillum rubrum* electron flow proceeds from the photochemical reductant through a special cytochrome, c_2, to the photochemical oxidant. In higher plant chloroplasts the addition of some sort of oxidation-reduction compound is necessary for the cycle to be completed at any appreciable rate (18, and see below).

The second pattern possible is shown in figure 2. An exogenous electron acceptor may be reduced, while a different compound is being oxidized. For instance in *Rhodospirillum* chromatophores, Frenkel has recently shown (19) that DPN may be reduced on the left, while $FMNH_2$ is oxidized on the right. It should be mentioned that this pathway did not lead to phosphorylation, although the completed cycle did. Smith and Baltcheffsky indicate, from spectrophotometric studies, that respiratory substrates may be the electron source on the right hand side (17). In this case the photochemical oxidant serves as an alternative to oxygen gas, for oxidation of the respiratory chain. Presumably pyridine nucleotides are the electron acceptors on the left, while ATP is made along the normal respiratory chain from the substrate (BH) on the right.

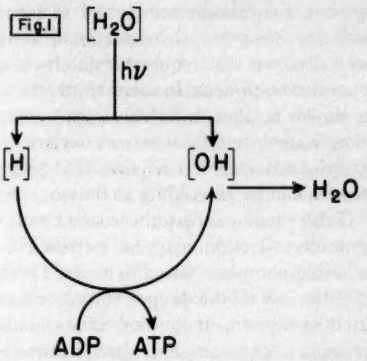
This 'U-shaped' pattern is of great importance to photosynthetic bacteria. These of course do not evolve oxygen, and must have some oxidizable substrate present to dispose of the extra photochemical oxidant produced in the light. In the over-all process carbon dioxide is the electron

---

[1] Contribution number 270 from the McCollum-Pratt Institute.

[2] Supported by Grant RG3923 from the National Institutes of Health, Research Grants Division, and in part by Grant G4379 from the National Science Foundation.

[3] This paper is not intended to be an exhaustive review of the field of photosynthetic phosphorylation. We have attempted to present the major trends without discussing in detail the work of all the laboratories now active in this area. Apologies should be made in advance, especially for inadequate coverage of phosphorylation by bacterial particles and for undue attention to our own data.



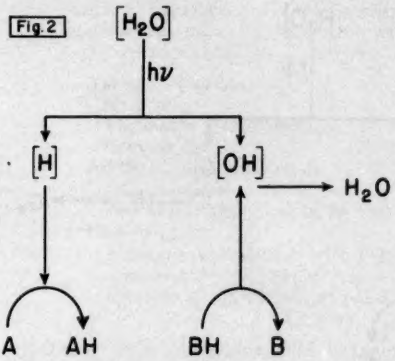


FIG. 1. Scheme for a cryptic electron transport cycle in chloroplasts. See text.
FIG. 2. Scheme for a light dependent coupled oxidation and reduction. See text.

acceptor on the left, and the photosynthetic substrates are the electron donors on the right hand side of this diagram.

The same pattern may also occur in chloroplasts, under the proper conditions. This was the interpretation placed on the reactions shown by Vernon and Kamen (20) in which ascorbic acid was oxidized (BH) while free oxygen gas served as the electron acceptor (A). Oxygen was reduced to hydrogen peroxide, which was trapped with ethanol and catalase. The reactions studied by Vernon and Hobbs (21) also fall into this category, with low potential oxidants as electron acceptors, and ascorbate or a mixture of ascorbate and indophenol dye as electron donors. Krogmann, working with Vennesland (22) has recently discovered phosphorylation associated with oxidation of reduced indophenol dye. Again, this may fit into the same pattern.

The final pattern is the one unique to green plants and algae, since these organisms evolve oxygen gas. In this sequence the photochemical oxidant is discharged by evolution of oxygen (figure 3). In the whole cell carbon dioxide is reduced; with isolated chloroplasts a large number of artificial oxidants can be reduced in the Hill reaction. Arnon, Whatley and Allen recently discovered phosphorylation associated with this pattern (23).

Our viewpoint is that the questions of greatest interest are going to concern the internal components of chloroplasts. From this point of view the sequence of electron transport through externally added cofactors, or Hill oxidants, is essentially a trivial matter. The function of exogenous oxidation-reduction cofactors is of interest, however, if it leads to a further understanding of the more complex internal pathways which are capable of generating high energy phosphate bonds.

HILL REACTION

In the Hill reaction, substrate amounts of the oxidant are reduced, stoichiometric with the amount of oxygen evolved. One of the most significant lines of research here was the demonstration by San Pietro and Lang (24) that deuterium atoms from heavy water are transferred to pyridine nucleotides, when these are Hill oxidants. More recently, Vishniac and Rose (25) have presented evidence suggesting that the hydrogen from water is transferred to chlorophyll itself in the light. They indicate further that this hydrogen may be transferred, in a second reaction, to TPN. As this research develops we may see direct evidence for chlorophyll as the first hydrogen carrier, and an intermediate between water and TPN.

Another factor quite definitely required with pyridine nucleotides is an easily extracted enzyme. With washed chloroplasts, or with fragments, hydrogen transfer from the chloroplast to TPN requires the addition of previously prepared soluble extract from chloroplasts. The enzyme has been highly purified by San Pietro (26), and given the name of 'photosynthetic Pyridine Nucleotide Reductase'. When purified it is specific for TPN. Kiester and San Pietro (personal communication) have recently found that the reduction of DPN requires another enzyme in turn. By independent tests the second enzyme was shown to be a transhydrogenase.

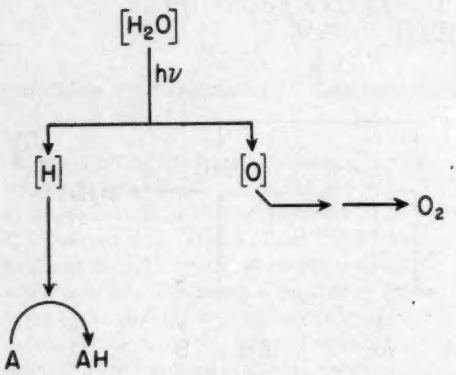


FIG. 3. Scheme for the Hill reaction. See text.

Any known Hill oxidant other than the pyridine nucleotides, however, can be reduced entirely without the need for PPNR.

Arnon and co-workers discovered that reduction of TPN by chloroplasts could support a simultaneous phosphorylation (23). In this case ATP is made in a stoichiometric relation to pyridine nucleotide reduction and oxygen evolution (note that this is the apparent reverse of the mitochondrial reactions in which pyridine nucleotides are oxidized and oxygen is consumed as ATP is generated). The stoichiometry approaches 1 mole of ATP formed per 1 mole of TPN reduced and ½ mole of oxygen evolved.

A likely site for this ATP formation is in the electron transport between the photochemical reductant and TPN. For this to be the case, however, we have to postulate a potential for the photochemical reductant considerably below −.32 volts; a situation not impossible to conceive. An alternative is for ATP formation to occur on the side of the photochemical oxidant, on the way from oxidant to oxygen gas. We think this is less likely, because with electron acceptors other than TPN or ferricyanide (see below), oxygen evolution occurs but does not lead to ATP formation. The difference between a phosphorylating and a non-phosphorylating Hill reaction in this case is most likely to occur on the reducing side, if only the electron acceptor has been changed.

The reduction of ferricyanide by chloroplasts is another reaction that can support ATP formation, again as discovered by Arnon and colleagues (23). We have looked at this phosphorylation and the accompanying electron transport in some detail (27). Not only does phosphorylation require a simultaneous reduction of ferricyanide; but also vice-versa, the reduction of ferricyanide at a maximal rate requires a simultaneous phosphorylation process. In other words the situation is similar to that in mitochondrial phosphorylation, where a maximal rate of oxygen uptake by healthy mitochondria requires that phosphorylation should be proceeding at the same time.

Table 1 shows that the control rate of ferricyanide reduction may be increased 3-fold by allowing phosphorylation to go on. There is some question as to the proper control value in this kind of experiment, in view of a stimulation by phosphate or magnesium alone, in the absence of ADP. However we feel that the most logical control is one which includes phosphate and magnesium but no ADP, and also some ATP which will be present very early and throughout the course of a phosphorylating reaction. Again this gives a rate almost equal to that of the original control.

The stoichiometry of this reaction, in our hands, varies from 0.8 to 1.2 μmoles of ATP formed per 2 μmoles of ferricyanide reduced—i.e. a P/2e ratio in the neighborhood of 1. However, if the ferricyanide reduced during a phosphorylating reaction includes some electron transport that is not making ATP, a truer measure of the efficiency of the reaction would be obtained by subtracting the basal rate. If this is done (28) the P/2e ratio in many experiments approaches 2. Unfortunately we have no inde-

TABLE 1. EFFECT OF PHOSPHORYLATING REAGENTS ON FERRICYANIDE REDUCTION

Additions	μmoles	Ferricyanide reduced	
		μM/mg chlorophyll/hr	% of control
None		240	100
Mg	10	333	139
P	25	320	133
Mg + P	10 + 25	400	166
Mg + P + ADP	10 + 25 + 1.0	750	310
ATP	0.2	170	71
Mg + P + ATP	10 + 25 + 0.2	216	90

Ferricyanide reduction measured by change of optical density at 420 mμ. Cuvettes contained, in 3 ml final volume: 40 μmoles of Tris pH 8, 70 μmoles of NaCl, 1.5 μmoles of ferricyanide, chloroplasts containing .030 mg. of chlorophyll, and other additions as shown. Optical density measured before and after a 2-minute exposure to light.

pendent way of determining whether it is valid to subtract the basal rate of ferricyanide reduction from the stimulated rate. A P/2e ratio of 2 for ferricyanide remains a speculation, therefore.

It is now of some interest to see that the phosphorylation that goes with TPN reduction is in some respects different from that accompanying ferricyanide reduction. TPN reduction, of course, require photosynthetic pyridine nucleotide reductase (PPNR), whereas ferricyanide reduction does not. A striking difference that we find very difficult to explain is that the reduction of TPN appears to be coupled only loosely to phosphorylation, whereas that of ferricyanide is fairly tightly coupled. That is, neither San Pietro nor we, nor Arnon have observed stimulation of the rate of TPN reduction by simultaneous phosphorylation. The significance of this is not clear to us; but it resembles the situation in some 'loosely coupled' mitochondria, or that induced by Lehninger's group with a protein extracted from mitochondria (29).

The third difference between TPN and ferricyanide relates to the pH optimum. Although both kinds of phosphorylation have a pH optimum in the neighborhood of 8, that with ferricyanide is extremely sharp compared to the optimum with TPN. At pH 7.2, TPN phosphorylation may be almost equal to that at 8.0, whereas ferricyanide phosphorylation would only be about 20% of that at 8.0 (unpublished observation).

We do not understand the meaning of these differences between TPN and ferricyanide phosphorylation and of course each may have its own individual explanation. However, their existence at least raises the possibility of two different kinds of phosphorylation sites, each with a slightly differing mechanism: one on the pathway to TPN, the other on the path to ferricyanide.

While ferricyanide reduction is fairly tightly coupled to phosphorylation, and TPN is loosely coupled to phosphorylation, reduction of other Hill reagents such as 2,3,6-trichlorophenolindophenol dye is definitely not coupled to phosphorylation. Thus David Korgmann when he was with us found that the rate of dye reduction by healthy chloroplasts was 3–5 times that of ferricyanide reduction (30). While ferricyanide reduction is stimulated 3 times by simultaneous phosphorylation, indophenol reduction is not substantially raised over its initially high rate. Finally no esterification of phosphate could be detected while indophenol was being reduced. Thus the reduction of this dye represents an inherently uncoupled electron flow.

TABLE 2. CHLOROPLAST PHOTOSYNTHETIC PHOSPHORYLATION, HILL REACTION WITH FERRICYANIDE AS ACCEPTOR

Not uncoupled by:
Calcium
Phosphate pre-incubation
Osmotic shock
Inhibited but not uncoupled by:
Dinitrophenol (1×10^{-3} M)
Pentachlorophenol (1×10^{-5} M)
Uncoupled by:
Arsenate (competitive with PO_4)
Ammonium ions (1×10^{-3} M)
Dilution in .35 M NaCl at pH 6

In chloroplasts, even more than in mitochondria, the internal electron transport components are not extractable into aqueous media. A bare beginning has been made into studies of lipid-extractable components by Lynch and French (31) and this has been carried on by Norman Bishop at the University of Chicago (32). Activity in ferricyanide and dye reduction is removed by a pretroleum ether extraction, and restored by re-addition of the extract in alcohol. In the most recent reports vitamin K_3 or coenzyme Q255, could replace the natural extract, (personal communication).

Having a coupled electron transport system, and (in the case of ferricyanide) being able to measure phosphate uptake and electron-flow simultaneously, we were naturally interested in uncouplers (28). Treatments which uncouple mitochondria, such as osmotic shock, or incubation with phosphate or calcium, are not effective with chloroplasts (table 2). Dinitrophenol we found to inhibit both electron flow and phosphorylation, to the same extent at the same concentration (and the concentration has to be high—10^{-3} M—for inhibition to occur). Pentachlorophenol is similarly, for chloroplasts, an inhibitor of coupled electron flow but not an uncoupler since it does not permit rapid electron flow in the absence of phosphorylation.

There are three ways that we have been able to induce uncoupled ferricyanide reduction in spinach chloroplasts. The first is a procedure, discovered by Krogmann (30), which involves diluting the chloroplasts considerably in .35 M NaCl at pH 6. The pH of 6 is a fairly sharp requirement, and essentially no uncoupling is seen at pH 7 or above. The salt concentration and

TABLE 3. EFFECT OF AMMONIUM SULFATE ON FERRICYANIDE REDUCTION AND ASSOCIATED PHOSPHORYLATION

Ammonium sulfate concentration, M	Ferricyanide reduced, μM		ATP formed, μM
	−ADP	+ADP	
0	0.21	0.59	0.36
1×10^{-4}	.35	.62	.32
2.3×10^{-4}	.57	.69	.20
3.3×10^{-4}	.62	.74	.12
1×10^{-3}	.67	.71	.02

Reaction conditions described in table 1.

dilution appear to be necessary for a rapid rate of uncoupling, but are not essential if you are willing to wait. We do not as yet understand the significance of these details of the procedure, which were discovered empirically.

The second way to induce uncoupled ferricyanide reduction is by adding ammonium ions to the reaction mixture. At about 10^{-3} M these stimulate electron flow greatly, while inhibiting ATP formation (28). Table 3 shows such an experiment. The effect is specific for ammonium ions, and is not shared by potassium, sodium, calcium or magnesium at these concentrations. The uncoupling is reversed when the chloroplasts are washed free of the ammonium salt.

Norman Good has recently completed a study of organic amines which he found to have the same effect as ammonium salts (personal communication). This ability appears to be shared by all monofunctional amines with a sufficiently short chain length.

The third way to cause uncoupled electron flow, as might be expected, is by using arsenate to replace phosphate in the phosphorylating reaction mixture (see below).

CYCLIC ELECTRON FLOW

As stated above, in bacterial particles the cycle may be completed entirely through enzymes endogenous to the extracted particles. In chloroplasts, however—at least, as they are isolated at present—endogenous enzymes are not sufficient to complete the gap from the photochemical reductant to the photochemical oxidant, except at very low rates.

The cyclic phosphorylation that proceeds without added cofactors seemed to be the most likely reaction to study when looking for possible internal enzymatic components of chloroplasts. Dr. Forti in our laboratory has been studying this 'endogenous' phosphorylation and finds even in this case that photosynthetic phosphorylation is not inhibited by antimycin A, amytal, or heptylquinoline-N-oxime. Thus with chloroplasts the usual sort of inhibitor studies provide us with no information relevant to a possible flavin-cytochrome b-cytochrome c type of electron transport sequence.

When external cofactors are added to the chloroplasts the cyclic electron transport proceeds at a very high rate. It is at the moment difficult to relate this cycle to reactions of the intact cell, especially since the discovery that ATP is made as pyridine nucleotides are reduced. While there is no direct evidence for the existence of a cycle in intact cells, recent studies by Arnon and his colleagues (33) show that carbon dioxide fixation by isolated and broken chloroplasts requires more ATP than can be provided during TPN reduction alone; that both Hill reaction and cyclic phosphorylation must be going on for carbon dioxide fixation to proceed all the way to sugars.

With isolated chloroplasts a very large number of miscellaneous dyes can serve as effective cofactors. Table 4 is a compilation of some of the compounds that we found effective (34). Arnon's group could add riboflavin (35) and miscellaneous vitamin K derivatives to the list (36), and Walker and Hill (37) have an even bigger list of synthetic dyes. Presumably any one of these is active, by itself, because it is first reduced by a coupled electron flow, then re-oxidized by the photochemical oxidant. These compounds deserve to be designated cofactors rather than coenzymes, because of the low specificity, and because no firm association has been shown for them with some specific protein.

Missing from this list is ascorbate, which was one of the three cofactors originally proposed by Arnon's group (15). It does not support appreciable phosphorylation by itself, and most laboratories working in the field seem to agree that it acts as a factor to stabilize the chloroplasts. Interest in ascorbate has been revived recently with the finding by Marrè of a TPNH-monodehydroascorbate reductase enzyme in whole chloroplasts, but not in chloroplast fragments (38).

Among the redox compounds acting as catalytic cofactors to complete the cycle, some interesting differences are apparent. The first one is that phenazine methosulfate (as shown here) and more especially its decomposition product

TABLE 4. COMPOUNDS THAT FUNCTION AS A REDOX-COFACTOR IN CYCLIC ELECTRON TRANSPORT

Compound	Conc.	Activity, μmoles P per mg chlorophyll per hr.	
		Exper. 1	*Exper. 2*
	μmoles/3 ml		
None		0	0
Phenazine methosulfate	0.06	735	555
FMN	0.10	158	123
Menadione	0.10	202	136
p-Phenylenediamine	0.20	15	31
Indigo carmine	0.20	175	159
Methyl viologen	0.10	156	110
Benzyl viologen	0.10	153	92

Reactions contained, in 3 ml volume: about .050 mg of chlorophyll, 40 μmoles of Tris, 70 μmoles of NaCl, 5 μmoles of phosphate, 8 μmoles of ADP, 10 μmoles of Mg Cl_2 and 0.1 μmole of the cofactor shown. Inorganic phosphate was measured at the beginning and end of the reaction.

pyocyanine (37) support phosphorylation at remarkably high rates; at least 3 times as high as with any other compound tested. The highest rates that we have observed in Baltimore are in the neighborhood of 900 μmoles of phosphate esterified per mg chlorophyll per hour (34). Dr. Avron, now at the Weizmann Institute in Israel, using Swiss Chard chloroplasts and extremely high light intensities, is able to observe rates of 1300–1400 μmoles per mg chlorophyll per hour (personal communication). It is significant that these very high velocities require a great deal of light—4000 foot candles for the 900 rate, and 12,000 foot candles for 1400 rate. These light intensities are comparable to those which saturate photosynthesis by almost any intact plant cell. Allen, Whatley and Arnon (39) have pointed out that a rate of 500 μmoles of phosphate per mg chlorophyll per hour is considerably faster than the best mitochondrial phosphorylations, when compared on an equal protein basis.

Another difference between cofactors, and possibly a very significant one, was the discovery of Walker and Hill (37) that phosphorylation by fresh chloroplasts with pyocyanine had a pH optimum at 7.0 as well as one at 7.7. FMN and K_3, on the other hand, have a sharp optimum at the higher pH. The least one can say is that pH 7.0 imposes a rate limitation with FMN that is not present with pyocyanin. The most one can say is that this may indicate the possibility of two different phosphorylation steps, one associated with pyocyanin and one associated with FMN. If this is a valid criterion, then ferricyanide fits with FMN mechanism, and TPN reduction with the pyocyanin mechanism.

Whatley, Allen and Arnon have segregated out an 'FMN pathway' from a 'vitamin K pathway' on the basis of a difference in sensitivity to dinitrophenol and to KCN (40). Under more primitive experimental conditions, Wessels has come to a somewhat similar conclusion (41). Whatley *et al.* indicate that phenazine methosulfate (which in white light is equivalent to pyocyanine) is like vitamin K, i.e. resistant to dinitrophenol and KCN, therefore phenazine methosulfate is put on the 'vitamin K pathway'. The correlation between vitamin K and pyocyanine is not yet complete, however, in that the pH 7 activity of vitamin K derivatives other than K_3 has not been explored fully yet. In general a complete picture of the possibly differing pathways possible with different cofactors must await a more systematic exploration of the presently available criteria with all of the compounds of interest, and possibly the discovery of more direct criteria.

We have investigated one difference between phenazine methosulfate and other catalytic cofactors in considerable detail ((42) and MS in preparation). We feel that we have been able to pinpoint this difference to the site of reoxidation of the cofactor, rather than to any difference in the phosphorylating site (fig. 5). The inhibitor CMU (p-chlorophenyldimethylurea, a weed-killer) is very effective against any Hill reaction, or against FMN or K_3 cycles. Figure 4, curve A, shows the sort of inhibition obtained with increasing concentrations of CMU in cyclic phosphorylation reaction mixture with FMN as cofactor. Fifty per cent inhibition occurs at about 3×10^{-6} M. However CMU has very little effect on phenazine methosulfate, when the latter compound is artificially reduced (either by the addition of ascorbate, or by a non-enzymatic photochemical anaerobic reaction in white light). Curve C shows this lack of effect. Pyocyanine, or phenizine methosulfate when not reduced artificially, show intermediate sensitivity (curve B).

Although there are complications here, especially when it comes to interpreting the intermediate sensitivities, the two extreme cases may be interpreted consistently as shown in figure 5. We postulate CMU to interrupt oxygen transport between water and oxygen gas. Specifically it

would do so after the first and before the second of at least two intermediates on this part of the oxygen transport pathway. Phenazine methosulfate, especially when completely reduced by ascorbate, can be oxidized by the internal component (the asterisk indicates prior reduction of the phenazine methosulfate by ascorbate or white light). CMU does not interfere with the formation of the first intermediate oxidant, and therefore it does not interfere with the cyclic electron transport running through phenazine methosulfate.

On the other hand we say that FMN or other components are not able to be oxidized by the first intermediate oxidant, but only by the next one in line. CMU interferes with the formation of the next one in line, and therefore with the re-oxidation of FMN. And of course, CMU blocks all oxygen evolution, and therefore any type of Hill reaction.

Dr. Margulies and I have gathered some further support for this scheme, in the form of experiments with the horseshoe-shaped reaction patterns (in preparation). For instance with ascorbate plus indophenol dye present to react with the first intermediate oxidant, we can show TPN reduction acquiring resistance to CMU inhibition.

Bové and Whatley, in Arnon's laboratory, have obtained almost an identical pattern of behavior for the inhibition seen with deficiency of chloride ions at a high pH (personal communication). Independently they have arrived at a scheme to account for the facts that is in most essentials similar to the one shown in figure 5.

Dr. Margulies has observed an inhibition of the Hill reaction and of phosphorylation by Black Valentine Bean leaf chloroplasts, if the bean leaves are stored at 0° C for about 24 hours in the dark (manuscript in preparation). Again, the pattern of reactions inhibited is identical to that with CMU as the inhibitor. Naturally we

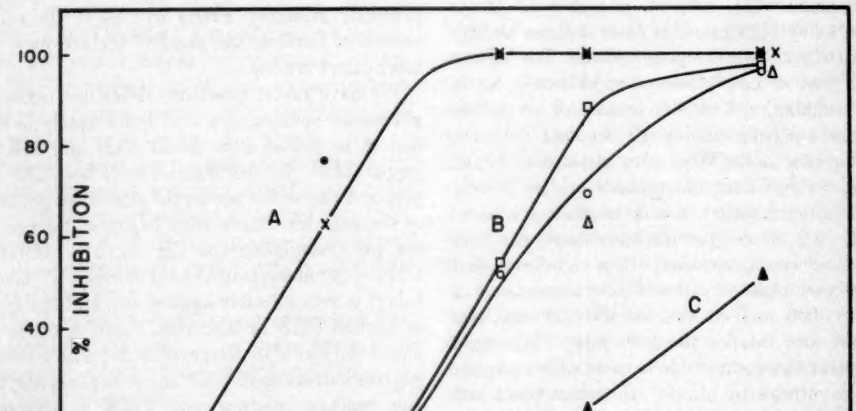


FIG. 4. Inhibition of photosynthetic reactions by p-chlorophenyl dimethylurea (CMU). Curve $A^{(x-x)}$ shows the % inhibition of phosphorylation with .1 μmole of FMN as a cofactor in 1.5 ml total volume. Curve A (●—●) shows the inhibition of indophenol dye reduction, measured as the change in optical density at 625 mμ in 3 ml reaction volume containing .010 mg of chlorophyll and .05 μmole of the dye. Curve B shows the inhibition of phosphorylation with .05 μmole of pyocyanine (□—□) or .05 μmole of phenazine methosulfate (PMS) as a cofactor, the reactions with PMS being run either in red light (○—○) anaerobically, or in white light in air (△—△). Curve C shows the CMU inhibition of phosphorylation with phenazine methosulfate as cofactor, using white light anaerobically. Per 1.5 ml reaction volume, all phosphorylation mixtures contained: 20 μmoles of Tris pH 8, 5 μmoles of $MgCl_2$, 5 μmoles of PO_4, 5 μmoles of ADP, and 35 μmoles of NaCl.

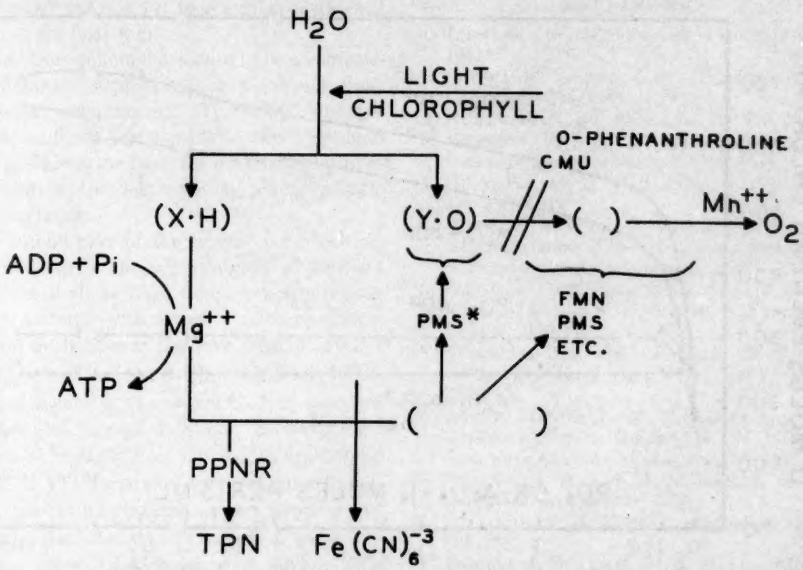


FIG. 5. Suggested integrated scheme for Hill reaction and for cyclic electron transport, resulting in phosphorylation. Site of inhibition by CMU or o-phenanthroline is shown as occurring between the first and second of 2 intermediates between H_2O and O_2. See text.

invoke the same inhibition site and the same reaction scheme to account for the observations.

Finally it should be noted that the site of inhibition for CMU has been postulated to occur at this same site in photosynthesis on the basis of experiments entirely different in nature, comparing the effect of the inhibitor on photosynthesis and on photoreduction by hydrogen adapted algae (43).

One final thing needs to be mentioned about current speculations on the nature of these electron transport cycles. Arnon, Whatley and Allen (44) observe a synergistic interaction between FMN and TPN plus photosynthetic pyridine nucleotide reductase, provided that FMN is present at a very low, sub-optimal concentration. On this basis they postulate TPN to be an intermediate hydrogen carrier before FMN. Two other observations lead us to be cautious about the significance of the observation, however. The first is that with these very low concentrations of FMN, a very considerable stimulation is also provided by oxygen (42), suggesting that the re-oxidation of FMN might be rate-limiting. The second is that with higher concentrations of FMN there is definitely no effect from adding TPN plus PPNR (44–46).

Preliminary observations of San Pietro and Giovanelli (personal communication) suggest that when a cycle is running through an optimal amount of FMN, there is no turnover of hydrogen in added TPN. This would be conclusive evidence that added TPN, although it stimulates with low FMN, is not an obligate intermediate at high levels of FMN.

MECHANISM OF ATP FORMATION

The evidence so far available with chloroplasts suggests that their mechanism for ATP formation is going to be at least partly different from that found in mitochondria. *Equations 1–6* show our hypothetical formulation for the mechanism of ATP formation, in the simple case of ferricyanide reduction by the Hill reaction (27).

$$H_2O + X + Y \rightarrow X\cdot H + Y\cdot O \quad 1$$
$$Y\cdot O \rightarrow Y + O_2 \quad 2$$
$$X\cdot H + I + A \rightarrow X + I^* + AH \quad 3$$
$$AH + Fe(CN)_6^{-3} \rightarrow A + H^+ + Fe(CN)_6^{-4} \quad 4$$
$$I^* + ADP \rightarrow I \backsim ADP \quad 5$$
$$I \backsim ADP + Pi \rightarrow I + ATP \quad 6$$

SUM: $H_2O + Fe(CN)_6^{-3} + ADP + Pi \rightarrow O_2 + Fe(Cn)_6^{-4} + H^+ + ATP$.

Note that we start out as usual with the photochemical oxidant and the photochemical reductant. The photochemical oxidant goes on to

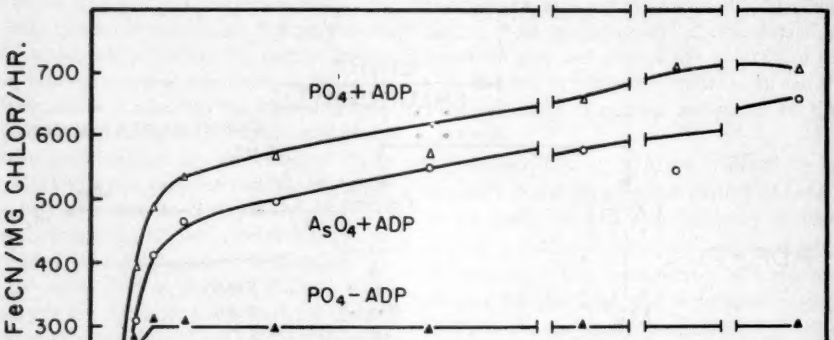


FIG. 6. Stimulation of ferricyanide reduction by phosphorylating reagents, and the substitution of arsenate for phosphate. Ferricyanide reduction was measured as described in table 1. In addition to chloroplasts, NaCl, Tris and ferricyanide, all reaction mixtures contained 10 μmoles of $MgCl_2$ and either phosphate or arsenate as shown. The two curves on top are from reaction mixtures containing 2 μmoles of ADP; those on the bottom are without ADP.

evolve oxygen gas (possible intermediates are ignored in *reaction 2*). The photochemical reductant is going to transfer its electrons to another intermediate carrier (designated 'A' in *reaction 3*), and ferricyanide gets reduced only in a subsequent step (*reaction 4*). At this point we cheerfully appropriated some of the concepts developed by Chance, Lehninger and others in connection with mitochondrial phosphorylations (48) and said that the transfer from initial photochemical reductant XH to 'A' is not a simple process, but involves a third component 'I'. In some unknown fashion 'I' is 'activated' to 'I*' as a result of the oxidation reaction in which it participates. The point about invoking the third entity is that its 'activated' state (perhaps an internal high energy bond) can be transferred to the high energy phosphate bond of ATP, as shown in *reactions 5 and 6*. Not only that, but we can arbitrarily call the activated form I* an inhibitor of *reaction 3* (or at least say that the unchanged I is required for *reaction 3* to proceed). In this case electron flow in *reaction 3* essentially will not proceed until the I* generated initially is degraded back to I. *Reactions 5 and 6* not only lead to the formation of ATP; they also cause I* to be converted back to I, and thereby permit more electron flow to occur in *reaction 3*. This then explains the coupled electron flow, in which ferricyanide reduction is greatly increased by allowing ATP formation to occur.

This set of reactions is highly speculative, and the only reactions for which we have any evidence are the last two (47). Specifically we think that ADP is involved in the formation of a high energy intermediate first, and the addition of phosphate is the last step in the process. This of course differs from the concepts in oxidative phosphorylation, where the addition of ADP is found to be the last step (49). Our evidence for this sequence in chloroplasts comes from the uncoupling effects of arsenate.

As seen in figure 6, in order for arsenate to act as an uncoupler ADP must be added to the reaction mixture (47). This fact implies the addition of arsenate to some pre-formed high energy ADP complex. If arsenate were to form a high energy intermediate first it would be expected to break down spontaneously. This hydrolysis in itself should lead to uncoupled electron flow, in the entire absence of ADP. But there is no uncoupling effect of arsenate without ADP, and the only way to visualize this is by the formation of an intermediate high energy ADP complex (I ~

ADP in our *reaction 5*), to which arsenate will add on as the final step.

In addition we found arsenate to be a competitive inhibitor for phosphate in the usual phosphorylation experiments. Therefore wherever arsenate adds on, one expects to find phosphate reacting. This is the basis for putting addition of phosphate as the last step in photosynthetic phosphorylation.

The second part of the scheme for which we have evidence is the irreversibility of the last step. First of all we have never seen appreciable ATPase activity, with active or inactive chloroplasts, in the light or in the dark, with or without the addition of various redox compounds, ADP, or any of a number of compounds that cause an inhibition of phosphorylation. Secondly the presence of arsenate does not induce arsenolysis of ATP. If ATP formation were reversible to any extent, one would certainly expect to see a hydrolysis of the intermediate formed in the reversal if an excess of arsenate were added. This would lead logically to a net breakdown of ATP in the presence of arsenate, which does not occur.

Finally, we have been unable to detect any significant amounts of exchange between ATP and inorganic phosphate, no matter which component was labeled, and again under a variety of conditions. Thus even the most sensitive reaction (exchange, rather than net breakdown) which might indicate reversibility of ATP formation is not detectable with chloroplast preparations.

In these details—the lack of ATPase, the lack of an exchange reaction, and the apparent reverse order of addition of ADP and phosphate—the chloroplast phosphorylation appears to differ from oxidative phosphorylation. Some extra support for this may be seen in the different spectrum of uncoupling agents that are effective in the two systems. Work in the future should provide additional insights into the similarities or differences between these two systems which synthesize ATP, and may give us more grounds for speculating on their evolutionary or ontogenetic relationships.

Any original work reported here represents to an equal extent the efforts of Dr. Mordhay Avron and myself, with major contributions from Dr. David W. Krogmann. Superior technical assistance was provided by Mrs. Marjory Evans for most of the work described.

REFERENCES

1. Lipmann, F. *Advances in Enzymol.* 1: 99, 1941.
1a. Lipmann, F. and L. C. Tuttle. *J. Biol. Chem.* 158: 505, 1945.
2. Ruben, S. *J. Am. Chem. Soc.* 65: 279, 1943.
3. Emerson, R. L., J. F. Stauffer and W. W. Umbreit. *Am. J. Botany* 31: 107, 1944.
4. Gest, H. and M. D. Kamen. *J. Biol. Chem.* 176: 299, 1948.
5. Wassink, E. C., J. E. Tjia and J. F. G. M. Wintermanns. *Koninkl. Akad. Wetenschap. Amsterdam. Proc. (C)* 52: 412, 1949.
6. Kandler, O. *Z. Naturforsch.* 5b: 423, 1950.
7. Simonis, W. and K. H. Grube. *Z. Naturforsch.* 7b; 194, 1952.
8. Strehler, B. L. *Arch. Biochem.* 43: 67, 1953.
9. Arnon, Daniel I. *Ann. Rev. Plant Physiol.* 7: 325, 1956.
10. Arnon, Daniel I., M. B. Allen and F. R. Whatley. *Nature, London* 174: 394, 1954.
11. Frenkel, A. *J. Am. Chem. Soc.* 76: 5568, 1954.
12. Newton, J. W. and M. D. Kamen. *Biochim. et biophys. acta* 25: 474, 1957.
13. Williams, A. M. *Biochim. et biophys. acta* 19: 570, 1956.
14. Thomas, J. B. and A. M. J. Haans. *Biochim. et biophys. acta* 18: 287, 1955.
15. Petrack, Barbara. *Fed. Proc.* 18 (1): 302, 1959.
16. Frenkel, Albert W. *J. Biol. Chem.* 222: 823, 1956.
17. Smith, Lucile and Margareta Baltcheffsky. *J. Biol. Chem.* 234: 1575, 1959.
18. Arnon, Daniel I., F. R. Whatley and M. B. Allen. *J. Am. Chem. Soc.* 76: 6324 (footnote 7a), 1954.
19. Frenkel, Albert W. *J. Am. Chem. Soc.* 80: 3479, 1958.
20. Vernon, L. P. and M. D. Kamen. *Arch. Biochem.* 51: 122, 1954.
21. Vernon, L. P. and M. O. Hobbs. *Arch. Biochem.* 72: 25, 1957.
22. Krogmann, D. W. *Biochim. et biophys. acta* 30: 655, 1958.
23. Arnon, Daniel I., F. R. Whatley and M. B. Allen. *Science* 127: 1026, 1958.
24. San Pietro, A. and H. M. Lang. *Science* 124: 118, 1956.
25. Vishniac, W. and I. A. Rose. *Nature, London* 182: 1089, 1958.
26. San Pietro, A. and Helga M. Lang. *J. Biol. Chem.* 231: 211, 1958.
27. Avron, M., D. W. Krogmann and A. T. Jagendorf. *Biochim. et biophys. acta* 30: 144, 1958.
28. Krogmann, D. W., A. T. Jagendorf and M. Avron. *Plant Physiol.* 34: 272, 1959.
29. Remmert, L. F. and A. L. Lehninger. *Proc. Nat. Acad. Sci. U. S.* 45: 1, 1958.
30. Krogmann, D. W. and A. T. Jagendorf. *Plant Physiol.* 34: 277, 1959.
31. Lynch, V. H. and C. S. French. *Arch Biochem.* 70: 382, 1957.

32. Bishop, N. I. *Proc. Nat. Acad. Sci. U. S.* 44: 501, 1958.
32a. Bishop, N. I. *Brookhaven Symp. in Biol.* 11: 332, 1959.
33. Arnon, D. I., A. V. Trebst and M. Losada. *Fed. Proc.* 18(1): 183, 1959.
34. Jagendorf, Andre T. and Mordhay Avron. *J. Biol. Chem.* 231: 277, 1958.
35. Whatley, F. R., M. B. Allen and Daniel I. Arnon. *Biochim. et biophys. acta* 16: 605, 1955.
36. Arnon, Daniel I., F. R. Whatley and M. B. Allen. *Biochim. et biophys. acta* 16: 607, 1955.
37. Hill, R. and D. A. Walker. *Plant Physiol.* 34: 240, 1959.
38. Marrè, E. and O. Arigoni. *Biochim. et biophys. acta* 30: 453, 1958.
39. Allen, M. B., F. R. Whatley and Daniel I. Arnon. *Biochim. et biophys. acta* 27: 16, 1958.
40. Whatley, F. R., M. B. Allen and D. I. Arnon. *Biochim. et biophys. acta* 32: 32, 1959.
41. Wessels, J. S. C. *Biochim. et biophys. acta* 25: 97, 1957; 29: 113, 1958.
42. Jagendorf, Andre T. and Mordhay Avron. *Arch. Biochem.* 80: 246, 1959.
43. Bishop, N. I. *Biochim. et biophys. acta* 27: 205, 1959.
44. Arnon, Daniel I., F. R. Whatley and M. B. Allen. *Nature, London* 180: 182, 1957.
45. Arnon, D. I., F. R. Whatley and M. B. Allen. *Biochim. et biophys. acta* 32: 47, 1959.
46. Avron, Mordhay and A. T. Jagendorf. *J. Biol. Chem.* 234: 1315, 1959.
47. Avron, M. and A. T. Jagendorf. *J. Biol. Chem.* 234: 967, 1959.
48. Chance, B. and G. R. Williams. *Advances in Enzymol.* 17: 65, 1956.
49. Wadkins, C. L. and A. L. Lehninger. *J. Biol. Chem.* 234: 681, 1959.

INTERCONVERSIONS OF HEXOSES

Saul Roseman

Rackham Arthritis Research Unit and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan

Recent work on the chemistry of complex polymers such as the mucopolysaccharides, mucoproteins, mucolipides, bacterial polysaccharides, endotoxins, etc., has shown that these substances contain large quantities of carbohydrate of varying monosaccharide composition, and that D-glucose is a relatively rare constituent. Some of the sugars found in these polymers, along with related substances, are presented in figure 1.

Although D-glucose is not a frequent component of the 'mucoid' substances, isotope studies reported by a number of laboratories have demonstrated that it is the precursor of the substances shown in figure 1, and that in each case the glucose carbon chain is preserved intact during its transformation to the new monosaccharide. Generally, the six carbon chain of D-glucose is converted directly to that of the six carbon chain of the hexose; thus, C-1 and C-6 of L-fucose are derived from C-1 and C-6 respectively of D-glucose. L-Ascorbic acid is unique in the sense that while it is also a product of glucose metabolism, the carbon chain is inverted so that C-1 of glucose yields C-6 of ascorbate, etc. In the plant, an alternate pathway for ascorbic acid metabolism also exists. The 9 carbon chain of neuraminic acid is formed from glucose; preliminary experiments indicate that the terminal six carbon atoms are derived directly from the glucose carbon chain, while the first three carbon atoms originate in some three carbon unit like pyruvate.

While the isotope studies suggested major pathways of metabolism, enzyme experiments have yielded more definitive information concerning the intermediates in the reaction sequences from D-glucose to the sugars of figure 1. Detailed reviews of recent work in these areas are now available (1, 2); the following discussion attempts only to outline the scope of information in this field.

Key substances in the interconversions of the hexoses are the sugar nucleotides which possess the general structure shown in figure 2; some examples of known compounds are indicated. The biosynthesis of specific sugar nucleotides involves one or more of three reaction types as shown in figure 3. *Reaction 1* represents the conversion of a nucleotide triphosphate (such as UTP or GTP) and a glycose-1-phosphate to a sugar nucleotide and inorganic pyrophosphate. In *reaction 2*, the glycose portion of the molecule is changed to another glycose by one or more of the indicated reaction types. *Reaction 3* has been

described only in the case of UDP-glucose and UDP-galactose and involves the transfer of a UMP residue from one glycose-P to another.

Examples of the reaction types are shown in

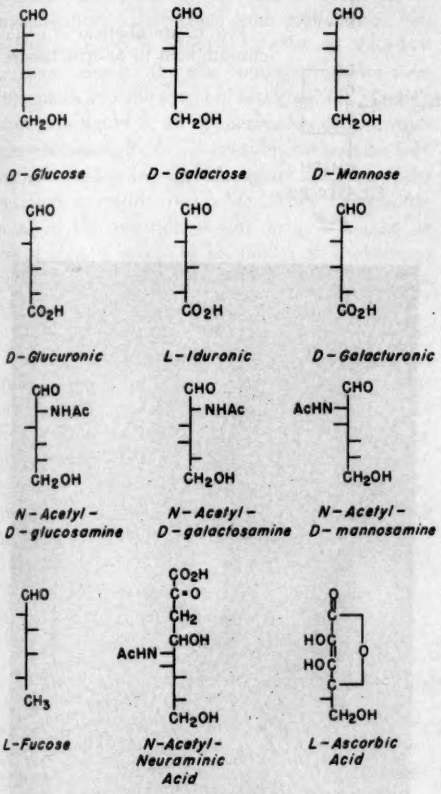
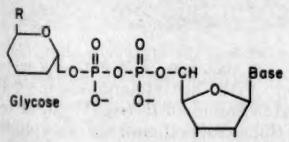


FIG. 1. Hexoses and related compounds.



R=			
CH_2OH	CO_2H	H	CH_3
Glucose	Glucuronic Acid	Xylose	
Galactose	Galacturonic Acid	Arabinose	
Acetylglucosamine			
Acetylgalactosamine			
Muramic Acid			
Mannose			Fucose

FIG. 2. Known sugar nucleotides. The double line separates the compounds containing uracil (above) from those containing guanine (below). The list is not intended to be complete, but indicates typical examples.

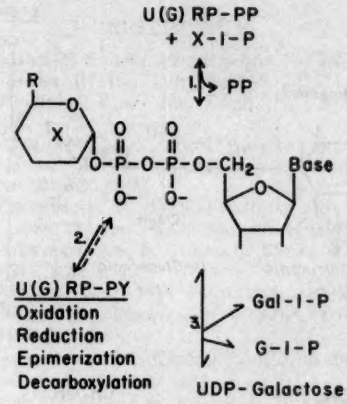
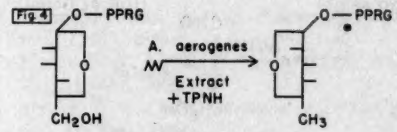


FIG. 3. Biosynthesis of sugar nucleotides. X and Y = different glycose residues; U = uracil; (G) = guanine; Gal-1-P = galactose-1-phosphate; G-1-P = glucose-1-phosphate.



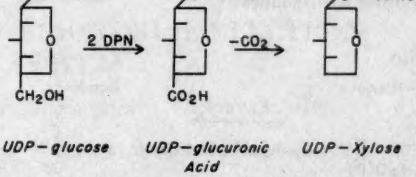


FIG. 4. Biosynthesis of GDP-fucose.

FIG. 5. Formation and decarboxylation of UDP-glucuronic acid.

figure 4, the synthesis of GDP-fucose (3) and in figure 5, the synthesis and decarboxylation of UDP-glucuronic acid (4, 5).

D-Glucuronic acid metabolism has been the subject of considerable work within the past few years. Some of the reactions of recent interest are shown in figure 6 (6–8).

Another group of sugars which has received attention are the hexosamines, characteristic constituents of the 'mucoid substances.' The amino sugar studied most intensively is D-glucosamine and its N-acetyl derivative; recent studies along these lines have been reviewed (1, 2). Much less is known about the metabolism of

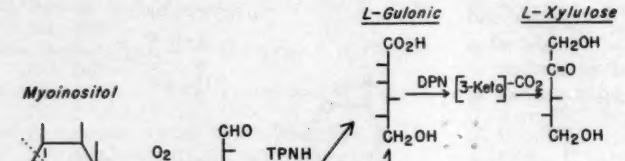


FIG. 6. Metabolism of D-glucuronic acid in animal tissues.

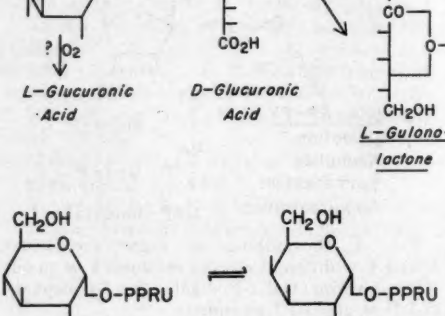


FIG. 7. Conversion of UDP-acetylglucosamine to other hexosamines.

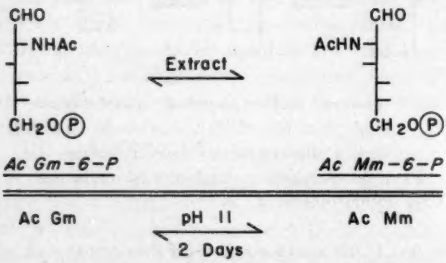


FIG. 8. Epimerization reactions. The reaction above the double line is catalyzed by a bacterial extract and involves the acetylhexosamine 6-phosphate esters. The reaction below the line is an alkaline epimerization of the acetylhexosamines.

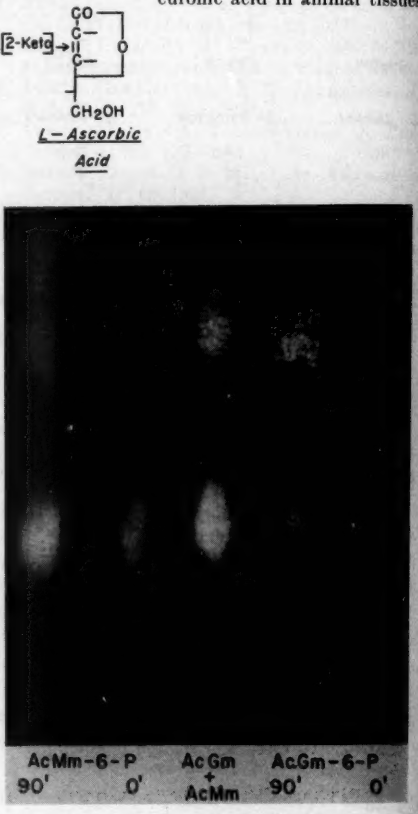


FIG. 9. Epimerase reaction. The extracts were incubated either with acetylmannosamine-6-P (left) or acetylglucosamine-6-P (right) for 0 or 90 min., the reactions stopped, and the phosphate esters hydrolyzed with potato phosphatase. The final reaction mixtures were de-ionized and chromatographed along with two standards (center).

D-galactosamine and D-mannosamine; in fact, the natural occurrence of the latter hexosamine was only recently discovered when it was shown that N-acetylneuraminic acid could be enzymatically cleaved to pyruvate + N-acetyl-D-mannosamine (9). The reaction was readily reversible, pointing a way towards the biosynthesis of certain of the sialic acids. As shown in figure 7, galactosamine and mannosamine derivatives are formed from UDP-acetylglucosamine (10–12). Whereas the epimerization of UDP-acetylglucosamine to UDP-acetylgalactosamine appears to be analogous to the interconversion of UDP-glucose and UDP-galactose, the conversion of UDP-acetylglucosamine to free N-acetylmannosamine seems

to be a unique biochemical reaction and the mechanism is not yet known.

Recent experiments in this laboratory indicate an alternate pathway for the biosynthesis of mannosamine derivatives from glucosamine derivatives. Bacteria adapted to grow on N-acetylmannosamine as the sole source of carbon were disrupted, and the extracts catalyzed the reaction shown in figure 8, an epimerization of N-acetylglucosamine-6-P to N-acetylmannosamine-6-P. As indicated in the chromatogram in figure 9, the reaction is readily reversible. While the mechanism of the reaction is unknown, it might be noted that it seems to be similar to a chemical epimerization of the acetylhexosamines which occurs at room temperature in alkali (13, 14).

To summarize this brief survey, therefore, considerable information is now available on the intermediary metabolism of some of the monosaccharides found in complex molecules whereas relatively little is known about the metabolism of others. In view of the increasing interest in these substances, however, it seems probable that progress on these problems will be very rapid indeed.

REFERENCES

1. Roseman, S. *Ann. Rev. Biochem.* 28: 545, 1959.
2. Holzer, H. *Ann. Rev. Biochem.* 28: 171, 1959.
3. Ginsburg, V. *J. Am. Chem. Soc.* 80: 4426, 1958.
4. Strominger, J. L., H. M. Kalckar, J. Axelrod and E. S. Maxwell. *J. Am. Chem. Soc.* 76: 6411, 1954.
5. Neufeld, E. F., D. S. Feingold and W. Z. Hassid. *J. Am. Chem. Soc.* 80: 4430, 1958.
6. Charalampous, F. C. and C. Lyras. *J. Biol. Chem.* 228: 1, 1957.
7. Bublitz, C., A. P. Grollman and A. L. Lehninger. *Biochim. et biophys. acta* 27: 221, 1958.
8. Ashwell, G., J. Kanfer and J. J. Burns. *Fed. Proc.* 17: 183, 1958.
9. Comb, D. G. and S. Roseman. *J. Am. Chem. Soc.* 80: 497, 1958.
10. Comb, D. G. and S. Roseman. *Biochim. et biophys. acta* 29: 653, 1958.
11. Glaser, L. *Biochim. et biophys. acta* 31: 575, 1959.
12. Maley, F. and G. Maley. *Biochim. et biophys. acta* 31: 577, 1959.
13. Roseman, S. and D. G. Comb. *J. Am. Chem. Soc.* 80: 3166, 1958.
14. Spivak, C. T. and S. Roseman. *J. Am. Chem. Soc.* 81: 2403, 1959.

ROLE OF LIPIDES IN THE MITOCHONDRIAL ELECTRON TRANSPORT SYSTEM

D. E. Green and R. L. Lester

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin

It has been known for more than a decade that lipide accounts for a sizable portion of the dry weight of mitochondria. Heart mitochondria, for example, contain about one fourth of their total dry weight in the form of lipide. There have been many hints that this lipide is vital to the structure and function of the mitochondrion. For example, mitochondria can be fragmented into less complex particulates and soluble enzymes by reagents such as bile salts, the lower alcohols and detergents—all of which either solubilize lipides in water or have marked solubility in both water and lipide. Then too, some of the most effective inhibitors of electron transport are lipide-like molecules such as antimycin A, heptyl-8-hydroxy quinoline N-oxide, and 2-hydroxynaphthoquinones substituted in the 3 position with alkyl nonpolar side chains (cf. Green (1) for summary of earlier work). In the last few years more concrete evidence has become available of the participation of lipides in electron transport and oxidative phosphorylation, and it is with these new developments that we shall be primarily concerned in the present review.

FORM OF MITOCHONDRIAL LIPIDE

In our own laboratory we became aware of the fact that the daughter particles resulting from the disruption of mitochondria varied in their lipide content from 0% to over 90%. This indicated that lipide was not randomly associated with all mitochondrial protein moieties—being concentrated only in certain segments of the mitochondrion. Up to the present, four lipide-rich

TABLE 1. KEY PROCEDURES IN ISOLATION OF LIPIDE OR LIPOPROTEIN FRACTIONS

Lipide Fraction or Lipoprotein	Starting Particle	Procedures	References
Q	SDC*	Cholate-KCl fractionation $(NH_4)_2SO_4$ fractionation	Basford and Green (2)
C_1	SDC	Deoxycholate-$(NH_4)_2SO_4$-Butanol fractionation Duponol treatment	Green *et al.* (4)
f_D	SDC Mitochondria	Cholate-KCl fractionation Petroleum ether extraction Gel formation in isooctane	Ziegler *et al.* (3)
lipid *c*	Mitochondria	Deoxycholate-$(NH_4)_2SO_4$ fractionation or heptane extraction	Widmer and Crane (5)

* SDC = succinic dehydrogenase complex.

fractions have been isolated from the mitochondria of beef heart muscle. We shall refer to these as the Q lipide fraction[1] (2), the f_D[2] lipoprotein (3), the cytochrome c_1 lipoprotein (4) and lipide cytochrome *c* (5). All but the last are water-soluble and form optically clear solutions in absence of bile salts and other solubilizing agents. They also have some solubility in organic solvents saturated with water such as butanol or ether. Lipide cytochrome *c* is soluble in organic solvents such as heptane and cyclohexane. When precipitated with ammonium sulfate the water-soluble lipide fractions form clear oils which usually rise to the surface during sedimentation. Freezing alters the structure of these lipide fractions as evidenced by the loss of water solubility. In table 1 are listed some of the key procedures which have been used in the isolation of the four lipide-rich fractions from various submitochondrial particles.

The amount of protein associated with each of the four lipide fractions varies widely. The protein content of the c_1 lipoprotein is 48%, of the f_D lipoprotein 15% and of the Q lipide fraction less than 1% if not zero. Lipide cytochrome *c* has not yet been sufficiently purified to permit a reliable estimate of protein content. The polypeptide content of these lipide fractions has yet to be explored. According to Basford, the Q lipide fraction contains a polypeptide which is released by treatment of the fraction with trichloracetic acid.

The f_D lipoprotein as isolated by the procedure of Ziegler *et al.* (3) is a complex of the DPNH dehydrogenase and a lipide moiety. The flavin content of the f_D lipoprotein is 14 mμmoles per mg protein while that of the homogeneous, lipide-free dehydrogenase is exactly the same. The ratio of flavoprotein to lipide at the highest purity level is constant from one preparation to another. In the ultracentrifuge the complex moves as a single component according to the unpublished studies of R. Bock and D. Gilboe.

The f_D lipoprotein can be stripped of lipide by various procedures, but in no case has it been possible to resolve the complex into a flavoprotein and lipoprotein. The lipide moiety invariably comes off as a water-insoluble, protein-free coagulum. The lipoprotein is then the complex between f_D and the lipide moiety.

The c_1 lipoprotein[3] can be resolved by Duponol into a lipide-free hemoprotein and a heme-free lipide fraction (4). Unlike the lipide of the f_D lipoprotein which is completely water insoluble the lipide of the c_1 lipoprotein can be detached in a water soluble form. The higher phospholipide content of the latter (*ca.* 80%) and the low content of the former (*ca.* 20%) may underlie this difference in behavior. The presence of minimal amounts of bile salts or other detergents may also be a factor in the solubilization of lipide.

The f_D lipoprotein has some enzymatic properties which are not shared by the lipide-free f_D. For example, the lipoprotein form of the dehydrogenase catalyzes the oxidation of over 400

[1] The Q lipide fraction has been previously designated as the interchain lipoprotein or Q lipoprotein (cf. Basford and Green, *ref. 2.*). With some modification of the original isolation procedure the fraction can be isolated in a form which contains less than 1% protein and which is indistinguishable catalytically from the form which contains 5% protein. Since the protein content of the Q lipide fraction can be reduced to negligible proportion without apparent sacrifice of function it would be misleading to continue to use the designation 'lipoprotein' for this fraction.

[2] f_D is an abbreviation for the flavoprotein associated with DPNH dehydrogenase activity.

[3] Main recognizable impurity in the cytochrome c_1 lipoprotein preparations made according to the method of Green *et al.* is flavin in the form of flavoproteins which may account for some 17% of the total protein.

μmoles of DPNH per minute per mg protein with ferricyanide as electron acceptor, whereas the lipide-free form has a specific activity of about 20 (3). It is of interest that mitochondrial-bound f_D shows the same high ferricyanide activity as f_D in the f_D lipoprotein—a fact which points to the association of f_D and lipide in the mitochondrion.

The Q lipide fraction as prepared by the procedures now followed in our laboratory contains so little protein that it is not justifiable to refer to this fraction as a lipoprotein (S. Fleischer, unpublished data). However, all the available evidence points to a highly ordered structure in this fraction, and it is therefore necessary to think of the Q lipide fraction as more than a mass of lipide. The fact of complete water solubility in absence of bile salts is alone sufficient proof of an ordered lipide structure. When the Q lipide fraction is treated with alcohol or frozen and thawed successively, the fraction loses its oriented structure and retrogrades into an amorphous precipitate of water-insoluble lipide. This change in state can be reversed by cholate or deoxycholate in low concentrations (R. L. Lester and S. Fleischer, unpublished data). The resolubilized lipide remains soluble in water even after the cholate is removed by exhaustive dialysis.

Coenzyme Q, the newly discovered member of the electron transfer chain (6), is found in the Q lipide fraction (2, 7). Neither the f_D lipoprotein nor the c_1 lipoprotein contain any measurable amount of coenzyme Q. A group of carotenoids, as yet unidentified, are also found exclusively in the Q lipide fraction.

The composition of the Q lipoprotein has been studied in detail by Basford (7), and the main lipide categories are listed in table 2. The bulk of the lipide is phospholipide of which almost half is in the form of plasmalogen. The phospholipide content of the Q lipide fraction is significantly different from that of the lipide of either mitochondria or of the c_1 and f_D lipoproteins (cf. table 3).

According to Crane and his co-workers cytochrome *c* exists in the mitochondrion in the form of a lipide complex which is water-insoluble (5). This complex can be converted or degraded into the water-soluble, classical form of cytochrome *c* by the action of phospholipase or butyl alcohol. The water-soluble cytochrome *c* which is extractable from mitochondria is probably an end result of the lipolytic fragmentation of lipide cytochrome *c*. Under appropriate conditions as summarized in table 1, lipide cytochrome *c* can be isolated from particles. But as yet no thorough study has been made of lipide *c*, and the purity of the preparations made thus far is still indeterminate.

TABLE 2. LIPIDE COMPONENTS OF Q-LIPIDE PREPARATION (7)

Neutral lipides (4.7% of total)	12% coenzyme Q 12% cholesterol carotenoids, triglycerides, etc.
Phospholipides (95.3% of total)	53% choline phosphatides 43% ethanolamine and serine phosphatides 3% inositol phosphatides

Aldehyde/phosphorus = 0.37.

TABLE 3. COMPOSITION OF VARIOUS LIPIDE-CONTAINING PREPARATIONS

Preparation	Lipide as % of dry weight	Phospholipide as % of total lipide*
Mitochondria	25	
f_D lipoprotein	85	<20
c_1 lipoprotein	52	80
Q lipide fraction	96–99	92–95

* Neutral lipide accounts for the rest.

The Q lipide fraction binds considerable amounts of various lipide-soluble compounds (cf. table 4) and also certain water soluble compounds like cytochrome *c* and silicomolybdate which can be converted to lipide-soluble forms. After interaction of water-soluble cytochrome *c* with the Q lipide fraction, a lipide cytochrome *c* is formed which is extractable into hydrocarbon solvents (2). Essentially all the component phospholipides of the Q lipide fraction form relatively stable water-insoluble complexes with cytochrome *c* (5).

The cytochrome oxidase which catalyzes the oxidation of reduced cytochrome *c* by molecular oxygen can be prepared in a form in which it shows essentially no catalytic activity unless supplemented with the Q lipide fraction or certain phospholipides thereof (8–10). The available evidence suggests that the true substrate for the cytochrome oxidase is a form of lipide cytochrome *c* (8). During purification of the enzyme the lipide content declines almost to zero, and at the final stage where the lipide content of the oxidase is minimal, the requirement for the Q lipide fraction is almost complete[4].

A requirement (in most cases absolute) for the Q lipide fraction has been established in assay

[4] Activity (μmoles reduced cytochrome *c* oxidized/min/mg protein) of this cytochrome oxidase preparation is approximately 0.7 without lipide supplement and 14–15 with the lipide supplement.

TABLE 4. COMPOUNDS BOUND BY Q-LIPIDE FRACTION (2)

Addition	Maximum amount taken up mμmoles/mg dry weight of Q lipide fraction
Silicomolybdate with Mg^+	121
Silicomolybdate without Mg^+	24
2,6-dichlorophenol-indophenol	5.5
Phenazine methosulfate	0.67
Cytochrome *c*	0.86
Vitamin K_1	0.6
Vitamin A acetate	2.0
d,α-Tocopherol	5.0
Coenzyme Q_{10}	25.0

systems for the following oxidoreductions: *1)* DPNH- cytochrome *c* reduction (Y. Hatefi and A. Haavik, unpublished observations); *2)* succinic-cytochrome *c* reduction (Lester and Fleischer, unpublished data); *3)* reduced coenzyme Q-oxygen reduction (11); *4)* succinic-oxygen reduction (Lester and Fleischer); *5)* reduced cytochrome *c*-oxygen reduction (8, 11). In some cases the Q lipide fraction can be replaced in part, if not wholly, by a purified single lipide component, but usually this substitution cannot be made. Neither the f_D nor c_1 lipoproteins can replace the Q lipide fraction in any of the above assay systems. However, lipide cytochrome *c* can replace the Q lipide fraction in the cytochrome oxidase assay system but its effect in any of the other assay systems has yet to be determined.

There are two alternative ways of interpreting the available data on the lipides of mitochondria. Either the lipide is distributed in discrete packets in various segments of the electron transfer chain—the combination of each packet with the adjacent protein corresponding to a lipoprotein, or the lipide exists in the form of a single entity which we may refer to as the lipide core without necessarily implying where the lipide is located within the mitochondrion. According to the first interpretation the lipoproteins are pre-existing entities, and the method of fragmentation of mitochondria and the isolation procedures lead to the separation of these entities. According to the second interpretation the lipide core is a continuum of a large number of lipides arranged in a definite pattern. The composition of any segment of the lipide core could be quite different from that of any other segment. The proteins which interdigitate with this lipide core are intimately bonded or complexed with specific segments of the core. Thus in the process of fragmenting the mitochondrion, segments of the lipide core become detached with the associated protein moieties. Thus multiple lipoproteins can be formed from the original single giant lipoprotein (lipide core plus the protein envelope).

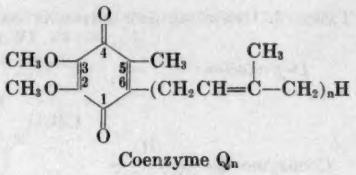
The available data are inadequate for deciding between these two alternative interpretations. The hypothesis of discrete, pre-existing lipoproteins was our first preference largely because the concept of a lipide core had yet to be recognized as a possibility. But as more information accumulates the idea of a lipide core is becoming more attractive as a working hypothesis.

It has to be assumed that there are lines of cleavage within the core which make possible the isolation of different segments of the core. For example, the procedure which leads to the isolation of cytochrome c_1 always detaches the same segment of the chain with which c_1 is normally complexed. The f_D and c_1 lipoproteins are derived by a cleavage process which leaves the protein still complexed with its corresponding segment of the lipide core. Lipide *c* may be derived simply by the cleavage of the segment of the lipide core in which cytochrome *c* is localized. The absence of coenzyme Q in the f_D and c_1 lipoproteins or in lipide *c* would be in line with the localization of components in the lipide core. This compartmentalization would account for the differences in composition of the four lipide fractions isolated thus far from mitochondria. Some oxidation-reduction proteins like DPNH dehydrogenase and cytochrome c_1 are more firmly attached to the lipide core than succinic dehydrogenase or cytochrome *a*. The former two can be readily isolated as lipoproteins while the latter two are more refractory in this respect.

The Q lipide fraction may be the closest approximation to the hypothetical lipide core in respect to size and composition. The physical measurements of R. Bock, D. Gilboe and R. Criddle (unpublished observations) suggest a molecular weight of 12–20 million for the Q lipide fraction. However, in the course of isolation of the Q lipide fraction, components such as lipide *c* may have been extracted and lost. Thus there would not be a 1:1 correspondence of the lipide composition of the Q lipide extract with that of mitochondrial lipide.

Whatever the uncertainty as to the arrangement and disposition of mitochondrial lipide the fact of a consistent association of various oxidation-reduction components with lipide of a defined composition, and the fact that these

protein-lipide complexes show enzymatic properties which are not necessarily shared by the corresponding lipide-free protein components are positive features of the problem. These lipide-protein complexes are providing new insight into the complexities of the electron transport process.



Coenzyme Q_n

FIG. 1.

COENZYME Q

Moore and Rajagopal of Cambridge in 1940 (12) were first to observe an alkali-labile component in the lipide extracts of rat liver with a band at 275 mμ. Later Morton and his colleagues at Liverpool independently confirmed the presence of this component in liver oils and other sources and concentrated the substance responsible for the 275 mμ band by chromatographic procedures (13). They isolated crystals with sharp melting points in the range of 33–41° (13–15). On the basis of chemical and spectroscopic properties they assigned a steroid structure to the compound—a position which they maintained in their various papers on the compound until late 1957 (14, 15). In June of that year Crane, Hatefi, Lester and Widmer of the Enzyme Institute announced the discovery and isolation of a quinone from mitochondria which they referred to as Q_{275} (6). This quinone was found to be concentrated in mitochondria from many sources and underwent oxidoreduction during active electron transport. The compound isolated by Crane *et al.* was in orange crystalline form with a sharp melting point of 48–49°. The yield from mitochondria was relatively high, and almost from the start, gm quantities of the crystalline compound were available. The method of isolation has recently been described in detail (16), and will not be considered further.

We then collaborated with the Merck Sharp & Dohme group led by Dr. Karl Folkers to determine the complete structure of the quinone. At that time the Enzyme Institute group were aware that the ring system which carried the quinone function was fully substituted and that there was a long polyisoprenoid chain in addition to two alkoxyl groups. The molecular weight according to crystallographic data and by redox titration was known to be about 900 (17, 18).

In some very elegant work Wolf *et al.* (19) of the Merck group established the complete structure of the quinone to be that shown in figure 1. The evidence which led to this assignment of structure is summarized in table 5. Nuclear magnetic resonance measurements were decisive in the detection of a methyl group in position 6 of the quinone nucleus and in categorizing essentially all of the hydrogen atoms in the molecule.

Lester, Crane and Hatefi in our laboratory had isolated coenzyme Q from a variety of sources and found evidence for homologues which shared the same quinonoid nucleus but differed in the number of isoprenoid units in the side chain (6, 16, 17, 20, 21). These natural homologues are listed in table 6. Coenzyme Q_6 from baker's yeast is the homologue with the smallest isoprenoid side chain that has been isolated thus far. Mammalian tissues in general contain coenzyme Q_{10}.

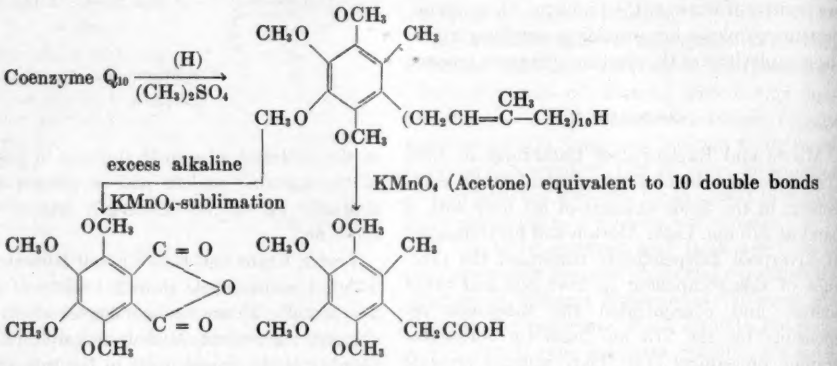
Shunk *et al.* (22) of Merck have prepared synthetically a wide variety of analogues and homologues of coenzyme Q, some of which are listed in table 7. These analogues and homologues will be considered in the context of the enzymatic properties of the coenzyme Q family of compounds.

Now we would like to return to the compounds originally isolated by Morton and his colleagues. Linn *et al.* (23) of the Merck Sharp & Dohme Research Laboratories in a recent note have concluded that the lower melting compounds originally isolated by the Liverpool laboratory were the mono- and diethoxy-derivatives of coenzyme Q_{10} or mixtures thereof, and that the original method of isolation led to the replacement of methoxy-groups by ethoxy-groups. The mono- and the diethoxy-Q_{10} are much less enzymatically active, and these were clearly artifacts of isolation.

After the note of Crane *et al.* had appeared, Morton *et al.* reported on the quinone nature of their original SA compound (14, 15) but in all subsequent work the quinone on which they have reported has the melting point of the quinone of Crane *et al.* (48–49°) and not that of their original SA compounds (33–41°). Strictly speaking, ubiquinone, the name which they have applied to the SA compound, should refer to the mono- and diethoxy-derivatives of coenzyme Q and not to the unmodified quinone.

TABLE 5. CHEMICAL AND PHYSICAL DATA BEARING ON THE STRUCTURE OF COENZYME Q_{10} (WOLF ET AL. (19))

Degradation:



NMR data:

Proton type	Number Found	Number Calculated
HC=	10	10
CH_3O—	6	6
=C—CH_2—CH=	2	2
=C—CH_2—CH_2—C=	40	36
CH_3C = (nucleus)		3
CH_3C = (chain)	33	33

TABLE 6. NATURAL HOMOLOGUES OF COENZYME Q ISOLATED IN PURE FORM (17)

Source	Number of Isoprenoid Units	Designation
Beef heart	10	Coenzyme Q_{10}
Torula utilis	9	Coenzyme Q_9
Azotobacter vinelandii	8	Coenzyme Q_8
Torula utilis	7	Coenzyme Q_7
Saccharomyces cerevisiae	6	Coenzyme Q_6

The Liverpool and the Hoffmann-LaRoche group in Basel have independently confirmed the complete structure of coenzyme Q_{10} first established by Wolf *et al.* of Merck Sharp & Dohme, and have synthesized coenzyme Q_6 (24, 25).

Now let us consider the coenzymatic properties of coenzyme Q. What is the experimental basis for assigning coenzyme function to Q? First, it is a normal component of mitochondria from a wide variety of sources—animal, plant and microbial (21, 26, 27). Secondly, when coenzyme Q is extracted from mitochondria, enzymatic activity is lost, and this activity can be specifically restored by addition of coenzyme Q (28, 30). Thirdly, it can be shown to undergo oxidoreduction within the mitochondrion at a rate compatible with a key role in the direct chain of the electron transport system (31–33). Lastly, inhibitors of electron transport affect the oxido-reduction state of bound Q in the manner anticipated (31, 32).

Lester and Fleischer have recently described a very simple way of extracting coenzyme Q from mitochondrial and submitochondrial particles (29). When the particles are extracted with dry acetone under specified conditions, coenzyme Q is rapidly and quantitatively extracted. The succinoxidase and succinic cytochrome *c* reductase activity of the extracted particles is lost and is restored to the original level by addition of coenzyme Q in relatively low amounts (Lester and Fleischer, unpublished data and ref. 29). In the succinoxidase assay both coenzyme Q and cytochrome *c* are absolute requirements, but one does not replace the other (cf. table 8).

The specificity of this effect of coenzyme Q is shown in table 9. Any of a considerable number of lipides with or without oxido-reduction properties are unable to duplicate the effect of coenzyme Q (28, 29, 34; also K. S. Ambe, F. L. Crane and W. F. Fechner, unpublished data).

Now let us compare the relative efficiency of

TABLE 7. HOMOLOGUES AND ANALOGUES OF COENZYME Q MODIFIED AT POSITION SIX AVAILABLE BY SYNTHESIS (SHUNK ET AL. (22))

Trivial Name	Substituent in Six Position
Coenzyme Q_0	H
Coenzyme Q_1	$-CH_2CH=\overset{CH_3}{\overset{\vert}{C}}-CH_3$
Coenzyme Q_2	$-(CH_2CH=\overset{CH_3}{\overset{\vert}{C}}-CH_2)_2H$
Coenzyme Q_3	$-(CH_2CH=\overset{CH_3}{\overset{\vert}{C}}-CH_2)_3H$
Heptyl Q	$-(CH_2)_6CH_3$
Phytyl Q	$-CH_2CH=\overset{CH_3}{\overset{\vert}{C}}-CH_2(CH_2CH_2\overset{CH_3}{\overset{\vert}{C}}HCH_2)_3H$
Heptadecyl Q	$-(CH_2)_{16}CH_3$
Eicosahydro Q_{10}	$-(CH_2CH_2\overset{CH_3}{\overset{\vert}{C}}HCH_2)_{10}H$
Aurantiogliocladin	$-CH_3$

coenzyme Q analogues and homologues in restoring the succinoxidase activity of acetone-extracted particles (Ambe *et al.* and ref. 34) (cf. table 10). From Q_1 to Q_{10} there is little to choose between one homologue and another. Furthermore analogues such as heptyl, phytyl and heptadecyl Q give lower but significant restoration. The dihydro-diacetyl derivative of Q_{10} is inactive (table 9) and the mono- and diethoxy derivatives have low activity (Lester and Fleischer). The succinoxidase activity induced by all the active compounds just mentioned is completely inhibited by antimycin[5].

If cytochrome *c* is used as the terminal electron acceptor rather than oxygen, basically the same specificity pattern emerges. Essentially only coenzyme Q homologues can catalyze the antimycin-sensitive reduction of cytochrome *c* by succinate (Ambe *et al.* unpublished data). But many quinones including some more polar analogues and homologues of coenzyme Q catalyze an antimycin-insensitive reduction of cytochrome *c* as shown in table 11. From this we may conclude that the specificity of Q homologues is sharper in the oxygen assay than in the cytochrome *c* reductase assay. The segment of the electron transport system responsible for the oxidation of reduced coenzyme Q by oxygen shows a higher degree of specificity for coenzyme Q homologues than does the segment of the chain responsible for the reduction of coenzyme Q. Only the coenzyme Q homologues can hurdle both specificity barriers.

An absolute requirement for coenzyme Q is

TABLE 8. REACTIVATION OF SUCCINOXIDASE ACTIVITY OF ACETONE EXTRACTED MITOCHONDRIA (29)

Additions	μatoms O_2/min/mg protein
None	0.00
Cyt. *c*	0.01
CoQ_{10}	0.05
CoQ_{10} + Cyt. *c*	0.53
as above + Antimycin	0.01
$CoQ_{10}H_2$ + Cyt. *c*	0.53

[5] In alternate experiments with isooctane extracted ETP, essentially the same specificity was observed in the restoration of succinoxidase. Various benzoquinone derivatives with the following substituents were tested and found inactive in these experiments: 2,3-dimethoxy; 2,3-dimethoxy,5,6-dimethyl; 2,5-dimethoxy,3-pentadecyl; 2,5-dimethoxy,3,6-dipentadecyl; 2,5-dimethoxy,3,6-diphytyl; 2,5-dihydroxy,3-pentadecyl; 2,5-dihydroxy,3,6-dipentadecyl; 2,6-dimethoxy. 2,5-Dimethoxy, phytyl benzoquinone, however, showed some trace activity.

TABLE 9. COMPOUNDS UNABLE TO REPLACE COENZYME Q IN RESTORATION OF SUCCINOXIDASE ACTIVITY IN ACETONE EXTRACTED PARTICLES

d-α-Tocopherol	Vitamin K_2 (C_{10}, C_{15}, C_{20} side chains)
Diacetyl-dihydro CoQ_{10}	2,3-Dimethoxy-5-methylbenzoquinone
Vitamin K_1	2,3-Dimethoxy benzoquinone
α-Tocopheryl quinone	Cholesterol
Benzohydroquinone	Chroman of CoQ_{10}
Serum albumin	Vitamin K—(*Mycobacterium phlei*)*
Tween 80	2,3-Dimethyl-1,4-naphthoquinone

* Generously provided by A. F. Brodie.

TABLE 10. RESTORATION OF SUCCINOXIDASE ACTIVITY IN ACETONE EXTRACTED-ETP BY VARIOUS COENZYME Q HOMOLOGUES*

Addition	Succinoxidase μatoms O_2/min/mg protein
None	0.00
Aurantiogliocladin	0.11
CoQ_1	0.52
CoQ_2	0.52
CoQ_3	0.50
CoQ_{10}	0.49
Phytyl Q	0.40
Heptyl Q	0.40
Heptadecyl Q	0.22
Menadione	0.03

* All assays in presence of cytochrome *c*. No activity in presence of antimycin. Unpublished data of K. S. Ambe, F. L. Crane and W. Fechner.

TABLE 11. RESTORATION OF SUCCINIC-CYTOCHROME *c* REDUCTASE ACTIVITY OF ACETONE EXTRACTED SDC*

Quinone Added	Succinic-Cyt. *c* Reductase Activity† (CoQ_{10} = 100)	Per cent Inhibition by Antimycin
None	0	
Aurantiogliocladin	30	0
CoQ_0	26	0
CoQ_1	50	20
CoQ_2	60	76
CoQ_3	69	100
CoQ_6	91	100
CoQ_{10}	100	100
Heptyl Q	50	0
Phytyl Q	57	86
Heptadecyl Q	15	100
Eicosahydro Q_{10}	38	100
$CoQ_{10}H_2$	100	100
Menadione	30	0

All compounds added at levels giving optimum response. Unpublished data of K. S. Ambe, F. L. Crane and W. Fechner.

* Prepared by method of Crane and Glenn (65).

† 100 = 0.6 μmoles succinate oxidized/min/mg protein.

demonstrable in the succinic-oxygen and succinic-cytochrome *c* systems and a partial requirement in the succinic-ferricyanide system. After acetone extraction the succinic-ferricyanide activity of particles falls to one third the original level—the level characteristic of the flavoprotein itself. On addition of Q this lost activity is restored, and the restored activity is fully antimycin-sensitive (30).

Coenzyme Q can be added back to acetone-extracted particles either as such or in the form of the Q lipide extract. The same maximal activity is reached regardless of the form in which the coenzyme is added (Lester and Fleischer, and ref. 35).

The DPNH-O_2 activity of acetone-extracted particles is not restorable by addition of coenzyme Q. Apparently, solvent extraction damages irreversibly the DPNH oxidase system. However, it is possible to demonstrate conclusively that coenzyme Q is also an essential component of the DPNH electron transfer chain. In mitochondria and several derivative particles, internal Q is rapidly reducible either by succinate or DPNH (31, 32). Complete reduction is achieved with either substrate. The rate of Q reduction by DPNH is exactly the same as or faster than the rate of oxidation of DPNH by oxygen or cytochrome *c* (33).

The reduction of internal coenzyme Q by succinate or DPNH is antimycin-insensitive while the oxidation of internal QH_2 by oxygen or cytochrome *c* is antimycin-sensitive (cf. Green *et al.* (33) and Pumphrey and Redfearn (36). The same applies to the reduction and oxidation of externally added coenzyme Q. Only the oxidation of externally added QH_2 is antimycin-sensitive (unpublished data of T. Ramasarma and R. L. Lester; and ref. 11). The rates of reduction and oxidation of exogenous coenzyme Q may equal but never has been found to exceed the rate of reduction and oxidation of internal coenzyme Q.

In particles capable of oxidative phosphorylation, Hatefi has shown that inorganic phosphate freezes bound internal Q in the reduced form while ADP transforms Q to the oxidized form (32). Exactly the same effects of phosphate and ADP are observed on the state of oxidoreduction of bound DPN and other electron carriers as shown in part by B. Chance and collaborators (37, 38). These observations establish that phosphorylation conditions determine the state of oxidoreduction of components in the chain, but

they do not necessarily implicate oxidized Q itself as an instrument of phosphorylation and dephosphorylation.

In general bacteria contain coenzyme Q (21). The family of mycobacteria is a notable exception. These contain no coenzyme Q but are rich in vitamin K. Animal mitochondria are rich in coenzyme Q, but there is no chemical evidence for the occurrence of vitamin K. There are some meso-organisms like *E. coli* and Azotobacter which contain both Q and K. It remains to be determined whether Q and K are both present in the same particles or whether there are two kinds or particles, one with a complement of Q and the other with a complement of K. According to the studies of Brodie *et al.* (39, 40) it would appear that in the particles of *Mycobacterium phlei* a vitamin K_1 compound fulfills a comparable role in electron transport as does coenzyme Q in animal mitochondria.

There have been reports by Beyer (41) and Dallam and Anderson (42, 43) that vitamin K can restore the phosphorylative activity of animal mitochondria which have been irradiated with light of 355 mμ. But it is difficult at present to assess the significance of these observations, as well as the previous work of Martius and coworkers (44, 45). In none of the systems where a vitamin K derivative has been shown to restore enzyme activity has it been demonstrated that the quinone undergoes oxidoreduction.

SOLVENT EXTRACTION OF PARTICLES

Nason and his colleagues at Johns Hopkins University introduced the immensely useful technique of extracting particles with organic solvents such as isooctane (46, 47). The use of organic solvents like isooctane and ether for the extraction of coenzyme Q from mitochondria and submitochondrial particles is not without some formidable complications. Residual traces of isooctane, e.g. as was shown by Pollard and Bieri (48, 49) as well as by several groups abroad (50, 51), inhibit the succinoxidase or cytochrome *c* reductase activity of particles, and this solvent inhibition is reversed by a variety of lipides including coenzyme Q as well as by removal of solvent by lyophilization or washing. The unspecific reversal of solvent inhibition should not be confused with specific restoration of activity which has been lost by the extraction of the component in question; when particles are extracted with isooctane sufficiently exhaustively to remove most of the bound coenzyme Q, then restoration of activity cannot be achieved with lipides other than coenzyme Q (6, 28–30).

The reversal of solvent inhibition by lipides appears to be a displacement of solvent molecules on the active sites of the particle by lipides. This is a unspecific process which can be duplicated by any of a large number of lipides. Nason *et al.* has reported a variety of experiments that demonstrate the capacity of α-tocopherol to restore the succinic-cytochrome *c* or DPNH-cytochrome *c* reductase activity of isooctane-extracted particles (52). According to Bieri and Pollard these results of Nason can be ascribed to the unspecific reversal of solvent inhibition by α-tocopherol (48, 49). Lester and Fleischer in our own laboratory (unpublished data) have found that acetone extracts 90% or more of the tocopherol content of mitochondria and yet find no requirement for α-tocopherol in the restoration of succinoxidase activity though an absolute requirement for coenzyme Q and cytochrome *c* is readily demonstrable. That α-tocopherol is present in mitochondria has clearly been demonstrated by Bouman and Slater (53) and by Donaldson and Nason (54), but the vitamin does not appear to undergo cyclical oxidation and reduction in the mitochondrion. In earlier determinations the levels of α-tocopherol were apparently overestimated (55).

Nason *et al.* (56, 57) have recently reported that in submitochondrial particles which have been aged, a requirement for α-tocopherol in the succinic-cytochrome *c* reductase assay emerges, and this requirement is highly specific. However, Bieri and Pollard contend that this is probably a reflection of the anti-oxidant activity of α-tocopherol rather than a catalytic effect (58). If aged particles deteriorate rapidly under assay conditions, α-tocopherol might merely be averting this rapid decline in activity. Tappel and Zalkin (59) have presented evidence for an antioxidant role of vitamin E in preventing the deleterious effects of lipide peroxidation on the activity of the succinoxidase and DPNH-cytochrome *c* reductase enzyme systems in mitochondria.

When particles are more exhaustively extracted with organic solvents to the point where not only Q but most of the lipide has been removed, the restoration of activity requires either a combination of Q and some suitable lipide supplement or the Q lipide fraction (unpublished data of Lester and Fleischer; also ref. 28). But

Q alone is inactive. Eventually, a point can be reached in the extraction where only the Q lipide fraction can restore activity, and thus far no combination of coenzyme Q and various lipide components can duplicate this effect (Lester and Fleischer). One has to distinguish between extracting coenzyme Q from the lipide of particles and extracting all the lipide. The former situation can be reversed by addition of Q while the latter requires reinsertion of a whole spectrum of lipides including coenzyme Q. There are all gradations between these two situations.

There is yet another parameter which is of importance to the problem of reintroducing coenzyme Q to an acetone-extracted particle. The particle may undergo some deformation in shape in consequence of extraction which makes it inaccessible to the external coenzyme. For example, the succinoxidase activity of acetone-extracted ETP[6] is not restored by coenzyme Q_{10} except in presence of a detergent such as lysolecithin or Triton. Coenzyme Q_2 effects restoration without any detergent being present (R. Bomstein, R. Goldberger and H. Tisdale, unpublished observations) Presumably because of molecular size, Q_{10} is incapable of penetrating the particle unless the particle is 'opened' by detergent action. The requirement for detergent applies not only to extracted particles but also to particles which have their full complement of lipide and coenzyme Q. The interaction of untreated particles with exogenous coenzyme Q or exogenous QH_2 is greatly enhanced in presence of detergents such as Triton, lysolecithin and fatty acid salts which both 'solubilize' the coenzyme and facilitate the entry of the coenzyme to the reactive sites (unpublished data Ramasarma and Lester; also D. E. Griffiths).

Lester and Fleischer have studied the specificity of the Q lipide extract in restoring the succinic-cytochrome *c* reductase activity of acetone-extracted mitochondria. None of the detergents (e.g. Triton, deoxycholate) nor individual lipide components (various phosphatides) that were tested in conjunction with coenzyme Q can restore an appreciable proportion of the lost activity. The Q lipide extract can be rendered water insoluble by several methods (freezing and thawing, extraction into solvents etc). These treatments lead to loss of activity. However by addition of bile salts the lipide can be resolubilized in a form which shows the same catalytic activity as the original or an appreciable part of the original activity. Loss of activity and water insolubility of the Q lipide extract appear to go parallel.

[6] ETP—Abbreviation for the electron transport particle (66).

VITAMIN K_1 IN ELECTRON TRANSPORT

Arnold Brodie and co-workers of Harvard (39, 40, 60, 61) have studied the electron transport and phosphorylation characteristics of subcellular particles of *Mycobacterium phlei*. In some very sharp experiments they have established the following cogent facts: *1*) these particles contain a vitamin K_1 derivative with about 30 atoms in the isoprenoid side chain; *2*) this K_1 derivative is destroyed by irradiation of the particles with light of wavelength 360 mμ; *3*) irradiated particles are inactive in catalyzing the oxidation of succinate by O_2 and the accompanying phosphorylation, but activity is fully restored by addition of vitamin K_1 (cf. table 12); *4*) coenzyme Q_{10} does not replace vitamin K_1 in this system just as vitamin K_1 or the naphthoquinone from *M. phlei* (table 3) does not replace coenzyme Q_{10} in the mammalian succinoxidase system. While coenzyme Q induces some succinoxidase activity, this activity is not coupled to phosphorylation.

Brodie, Davis and Fieser (40) have purified from *Mycobacterium phlei* a naphthoquinone which they have partially characterized as a vitamin K_1 type compound according to the data given in table 13. It is interesting to note that a similar compound had been previously purified by Snow

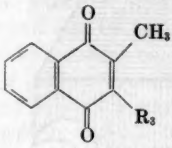
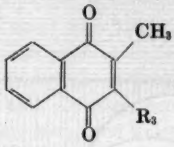
TABLE 12. RESTORATION OF OXIDATIVE PHOSPHORYLATION BY VITAMIN K_1 AND M. PHLEI NAPHTHOQUINONE

System	Additions	Oxidation† (μatoms)	ΔPi† (μmoles)	P/O
Untreated particles + supernatant	none	3.0	2.7	0.9
	Vit K_1 (1 μmole)*	3.8	2.9	0.77
Irradiated particles (360 mμ) + supernatant	none	0.0	0.0	0.0
	Vit. K_1 (1 μmole)	3.16	3.0	0.95
	M. phlei naphthoquinone (0.1 μmole)	1.56	2.1	1.35
	Coenzyme Q (0.8 μmole)	0.21	0.0	0.0

* μmoles/ml.

† Experiment carried out at 30° for 10 min. Data of A. F. Brodie.

TABLE 13. COMPARISON OF M. PHLEI NAPHTHOQUINONE WITH VITAMIN K_1

	Vitamin K_1	*M. Phlei Naphthoquinone*
Nucleus		
Side chain (R_3)	C_{20} (phytyl)	C_{25}–C_{35}
Molecular weight	450	$\leqslant$620
IR spectra		same as K_1
U.V. spectra		same as K_1 or K_2
R_f	0.17	0.06
Conc. in *M. phlei* particles	none	9.3 μmoles/g protein

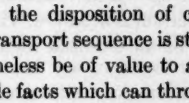
Data of Brodie *et al.* (40).

(62) and crystallized by Noll (63) from the lipide of *Mycobacterium tuberculosis* (Brevannes). Noll characterized this compound as a 2-methyl-1,4-naphthoquinone derivative with a K_2-type side chain at position three, with either 7 or 8 isoprenoid units (63).

STRUCTURE AND SEQUENCE OF THE ELECTRON TRANSPORT CHAIN

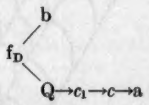
The large amount of mitochondrial lipide, the existence of at least two lipide-soluble oxidation-reduction components of the electron transport chain, and the close association of cytochrome c_1 and f_D with lipide moieties are only a few of the many clear indications that lipide goes to the heart of the electron transport process. Although knowledge of the disposition of components in the electron transport sequence is still incomplete, it may nonetheless be of value to assemble some of the available facts which can throw light on the role of lipide in electron transport.

Electrons can enter the mitochondrial chain either by way of succinate or DPNH (bound in the case of the mitochondrion and free in the case of submitochondrial particles). According to Green, Ziegler and Doeg (64) the sequence of the succinic chain is the following:



When succinate is oxidized by the flavoprotein with succinic dehydrogenase activity (f_S) the two electrons released are transferred to the flavin of the dehydrogenase. Then a choice is possible—either transfer to cytochrome b[7] or to coenzyme Q. From coenzyme Q to oxygen the intermediates are cytochromes c_1 and c. From cytochrome b to oxygen, the pathway is still indeterminate but the possibility of a link between cytochrome b and coenzyme Q cannot be ruled out.

The sequence for electrons from DPNH to oxygen is probably very similar, if not identical, with that for the succinic chain:



The link of f_S with b (and by analogy the link of f_D with b) does not involve lipide, and cytochrome b can readily be isolated in a lipide-free form. By contrast, from flavoprotein to cytochrome a in the segment involving coenzyme Q, and cytochromes c_1 and c, lipide is involved from one end to the other. This segment is the most logical candidate for the lipide core which was postulated under first heading. The fact that all the hemes and coenzyme Q are reducible by either succinate or DPNH even though the succinic and DPNH chains have separate complements of cytochrome b, strongly suggests that the lipide core is shared by the two chains in a manner suggested by the diagram in figure 2.[8] The lipide core has at least two compartments—one in which coenzyme Q is localized and one in which lipide c is localized. These compartments apparently do not intercommunicate. Cytochrome c_1 is the antimycin-sensitive link between these two compartments, i.e. between QH_2 and cytochrome c. Furthermore, the details of the sequence by which electrons from reduced cytochrome b reach cytochrome a are largely unknown. However, it is clear that there are two

[7] According to unpublished studies of D. M. Ziegler and K. A. Doeg protoheme is one of the functional groups of succinic dehydrogenase and thus the succinic dehydrogenase accounts for part of the cytochrome b fraction.

[8] For sake of simplicity the idea of a common lipide core shared by the succinic and DPNH chains has been favored in the context of the present review. It cannot be excluded however that there is a separate lipide core associated with each of the two chains. This is indeed a more likely possibility than the single lipide core hypothesis.

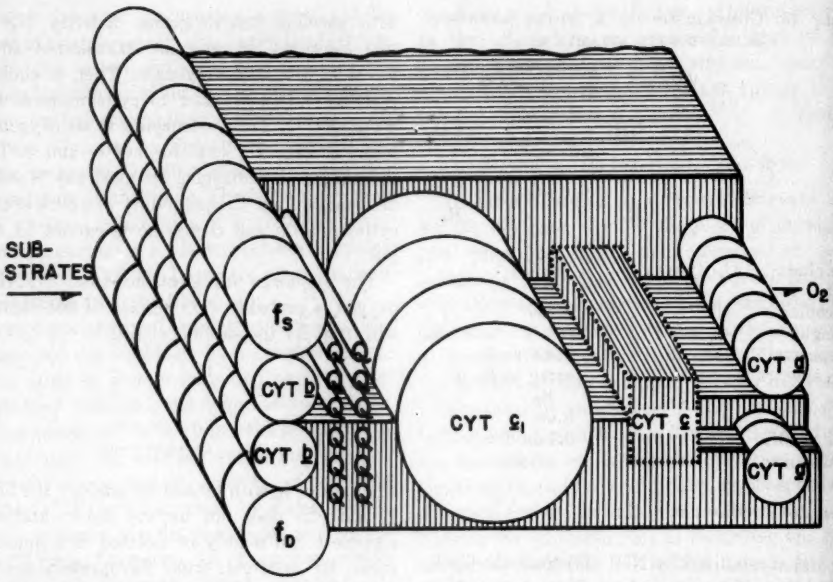


FIG. 2. Schematic representation of electron transport chain indicating possible distribution of lipide (shaded area) and various known electron carriers (unshaded areas). The possible polymeric nature of the structure is indicated in the third dimension. For the sake of simplicity other known enzymes intimately associated with electron transport reactions (citric acid cycle enzymes, fatty acid oxidation enzymes, etc.) are omitted. As indicated in the text, which electron carriers interact directly with cytochrome *b* is still obscure.

pathways for electrons *1*) by way of the lipide core and cytochrome c_1; and *2*) by way of cytochrome *b* and the corresponding chain. These pathways could be alternative or parallel. The indications are that in phosphorylating mitochondria the pathways are parallel and of equal importance whereas in non-phosphorylating particles the flux of electrons is predominantly through the lipide core and cytochrome c_1.

The lipide core is a non-aqueous medium, and thus at least four oxidoreductions can take place in virtual absence of water (reduced flavoprotein with Q, QH_2 with cytochrome c_1, reduced cytochrome c_1 with cytochrome *c*, and reduced cytochrome *c* with cytochrome *a*). Two of the five participants, viz coenzyme Q and lipide *c* are known to be localized in the lipide core while the other three, viz the flavoprotein and cytochromes c_1 and *a*, are intimately associated with the lipide core. Probably the functional groups of these three proteins penetrate into the lipide core.

Coenzyme Q and lipide *c* are probably mobile within the lipide core and can thus shuttle electrons from reduced flavin to c_1 and from reduced c_1 to cytochrome *a*. The molecular weights of coenzyme Q and cytochrome *c* are small, relative to that of the components which are being linked (863 and 13,000 respectively, compared to 200,000 and above).

If, as seems probable, the oxidoreductions in the non-aqueous medium of the lipide core are coupled to esterification of inorganic phosphate, then the meaning of mitochondrial lipide lies in the mechanism by which coupling of oxidation and phosphorylation is achieved. The elimination of water may be an essential ingredient in this mechanism.

POLYMOLECULAR STRUCTURE OF COMPONENTS OF ELECTRON TRANSPORT CHAIN

The diagrams representing the disposition of components in the electron transport chain show any one component, e.g. cytochrome c_1, linked functionally to at least two other components in the chain. It has to accept electrons from reduced Q and donate electrons to oxidized cytochrome *c*. At the same time the rigidity of the particle precludes any major molecular oscillation or translation of cytochrome c_1. A lipide-free form of cytochrome c_1 has been isolated in homoge-

neous state by Green *et al.* (4) and shown by Criddle and Bock (67) to have five heme groups per *m* unit of 370,000. Thioglycollate in the presence of a detergent depolymerizes the polymolecular unit into the basic units of *m* 70,000, each of which contains only one mole of heme per mole of protein. The polymolecular unit can be arranged in such a way that electrons can enter the unit through one heme group and leave by way of another. Moreover, intercommunication within the unit through metals or other devices in effect make cytochrome c_1 an electronic crossroad in the electron transport sequence.

Cytochrome *a* has been shown by Ambe and Venkataraman (8) to exist in a polymolecular form which is particulate. On treatment with thioglycollate, this particulate unit is depolymerized to a water-soluble form of cytochrome *a* of molecular weight about 72,000, containing 1 molecule of cytochrome *a* per molecule of protein. The number of cytochrome *a* molecules in each polymolecular unit cannot be ascertained because of the water insolubility of the unit.

If the smallest common denominator for the f_D lipoflavoprotein were a single molecule of flavoprotein linked to a lipide the minimal molecular weight would be 490,000 ($74{,}000 \times \frac{100}{15}$) (3). Lipide accounts for about 85% of the total dry weight of the f_D lipoflavoprotein. The ultracentrifuge data of Bock and Criddle suggest that the observed molecular weight is some multiple (at least 4) of the minimal molecular weight.

There are thus at present three authentic examples of a polymeric structure for oxidation-reduction components of the electron transport chain. It is highly probable that this is a general feature of the arrangement of all electron transport components. Figure 2 is a three dimensional representation of the electron transport chain which emphasizes the polymolecular character of the chain. The 'native' polymers may be much larger than the isolated polymeric forms. Perhaps there is a selection process in the isolation procedure which favors the low molecular weight polymers.

REFERENCES

1. Green, D. E. *Advances in Enzymol.* 21: 73, 1959.
2. Basford, R. E. and D. E. Green. *Biochim. et biophys. acta* 33: 185, 1959.
3. Ziegler, D. M., D. E. Green and K. A. Doeg. *J. Biol. Chem.* 234: 1916, 1959.
4. Green, D. E., J. Järnefelt and H. D. Tisdale. *Biochim. et biophys. acta* 31: 34, 1959.
5. Widmer, C. and F. L. Crane. *Biochim. et biophys. acta* 27: 203, 1958.
6. Crane, F. L., Y. Hatefi, R. L. Lester and C. Widmer. *Biochim. et biophys. acta* 25: 220, 1957.
7. Basford, R. E. *Biochim. et biophys. acta* 33: 195, 1959.
8. Ambe, K. S. and A. Venkataraman. *Biochem. Biophys. Res. Communications* 1: 133, 1959.
9. Greenlees, J. and W. W. Wainio. *J. Biol. Chem.* 234: 658, 1959.
10. Hatefi, Y. *Biochim. et biophys. acta* 30: 648, 1958.
11. Hatefi, Y. *Biochim. et biophys. acta* 34: 183, 1959.
12. Moore, T. and K. R. Rajagopal. *Biochem. J.* 34: 335, 1940.
13. Festenstein, G. N., F. W. Heaton, J. S. Lowe and R. A. Morton. *Biochem. J.* 59: 558, 1955.
14. Morton, R. A., G. M. Wilson, J. S. Lowe and W. M. F. Leat. *Chem. & Industr.* 1649, 1957.
15. Morton, R. A., G. M. Wilson, J. S. Lowe and W. M. F. Leat. *Biochem. J.* 68: 16P, 1958.
16. Crane, F. L., R. L. Lester, C. Widmer and Y. Hatefi. *Biochim. et biophys. acta* 32: 73, 1959.
17. Lester, R. L., F. L. Crane and Y. Hatefi. *J. Am. Chem. Soc.* 80: 4751, 1958.
18. Lester, R. L., Y. Hatefi, C. Widmer and F. L. Crane. *Biochim. et biophys. acta* 33: 170, 1959.
19. Wolf, D. E., C. H. Hoffman, N. R. Trenner, B. H. Arison, C. H. Shunk, B. O. Linn, J. F. McPherson and K. Folkers. *J. Am. Chem. Soc.* 80: 4752, 1958.
20. Lester, R. L. and F. L. Crane, *Biochim. et biophys. acta* 32: 492, 1959.
21. Lester, R. L. and F. L. Crane. *J. Biol. Chem.* 234: 2169, 1959.
22. Shunk, C. H., B. O. Linn, E. L. Wong, P. E. Wittreich, F. M. Robinson and K. Folkers. *J. Am. Chem. Soc.* 80: 4753, 1958.
23. Linn, B. O., N. R. Trenner, C. H. Shunk and K. Folkers. *J. Am. Chem. Soc.* 81: 1263, 1959.
24. Morton, R. A., U. Gloor, O. Schindler, G. M. Wilson, L. H. Chopard-dit-Jean, F. W. Hemming, O. Isler, W. M. F. Leat, J. F. Pennock, R. Rüegg, U. Schwieter and O. Wiss. *Helv. Chim. Acta* 41: 2343, 1958.
25. Gloor, U., O. Isler, R. A. Morton, R. Rüegg and O. Wiss. *Helv. Chim. Acta* 41: 2357, 1958.
26. Page, A. C., B. O. Linn, P. H. Gale, E. L. Wong, C. H. Shunk and K. Folkers. *Fed. Proc.* 18: 297, 1959.
27. Morton, R. A., *Nature, London* 182: 1764, 1958.
28. Crane, F. L., C. Widmer, R. L. Lester and Y. Hatefi. *Biochim. et biophys. acta* 31: 476, 1959.
29. Lester, R. L. and S. Fleischer. *Arch. Biochem.* 80: 470, 1959.

30. Crane, F. L., W. Fechner and K. S. Ambe. *Arch. Biochem.* 81: 277, 1959.
31. Hatefi, Y., R. L. Lester, F. L. Crane and C. Widmer. *Biochim. et biophys. acta* 31: 490, 1959.
32. Hatefi, Y. *Biochem. et biophys. acta* 31: 502, 1959.
33. Green, D. E., Y. Hatefy and W. F. Fechner *Biochem. Biophys. Res. Communications* 1: 45, 1959.
34. Crane, F. L., C. H. Shunk, F. M. Robinson and K. Folkers. *Proc. Soc. Exper. Biol. & Med.* 100: 597, 1959.
35. Fleischer, S. and R. L. Lester. *Fed. Proc.* 18: 226, 1959.
36. Pumphrey, A. M. and E. R. Redfearn. *Biochem. J.* 72: 2, 1959.
37. Chance, B. and G. R. Williams. *Advances in Enzymol.* 17: 65, 1956.
38. Chance, B. and M. Baltscheffsky. *Biochem. J.* 68: 283, 1958.
39. Brodie, A. F., M. M. Weber and C. T. Gray. *Biochim. et biophys. acta* 25: 448, 1957.
40. Brodie, A. F., B. R. Davis and L. F. Fieser. *J. Am. Chem. Soc.* 80: 6454, 1958.
41. Beyer, R. F. *Biochim. et. biophys. acta* 28: 663, 1958.
42. Dallam, R. D. and W. W. Anderson, *Biochim. et biophys. acta* 25: 439, 1957.
43. Anderson, W. W. and R. D. Dallam. *J. Biol. Chem.* 234: 409, 1959.
44. Martius, C. and D. Nitz-Litzow. *Biochim. et biophys. acta* 13: 152, 1954.
45. Martius, C. and D. Nitz-Litzow. *Biochim. et biophys. acta* 13: 228, 1954.
46. Lehman, I. R. and A. Nason. *J. Biol. Chem.* 222: 497, 1956.
47. Nason, A. and I. R. Lehman. *J. Biol. Chem.* 222: 511, 1956.
48. Pollard, C. J. and J. G. Bieri. *Biochim. et biophys. acta* 30: 658, 1958.
49. Pollard, C. J. and J. G. Bieri. *Biochim. et biophys. acta* 34: 420, 1959.
50. Deul, D., E. C. Slater and L. Veldstra. *Biochim. et biophys. acta* 22: 133, 1958.
51. Weber, F., U. Gloor and O. Wiss. *Helv. Chim. Acta* 41: 1038, 1958.
52. Nason, A., K. O. Donaldson and I. R. Lehman. *Trans. N. Y. Acad. Sci.* 20: 27, 1957.
53. Bouman, J. and E. C. Slater. *Biochim. et biophys. acta* 26: 624, 1957.
54. Donaldson, K. O. and A. Nason. *Proc. Nat. Acad. Sci. U. S.* 43: 364, 1957.
55. Bouman, J., E. C. Slater, H. Rudney and J. Links. *Biochim. et biophys. acta* 29: 456, 1958.
56. Donaldson, K. O., A. Nason, K. B. Moore and R. H. Garrett. *Biochim. et biophys. acta* 26: 665, 1957.
57. Vasington, F., K. O. Donaldson and A. Nason. *Fed. Proc.* 17: 327, 1958.
58. Pollard, C. J. and J. G. Bieri. *J. Biol. Chem.* In press.
59. Tappel, A. L. and H. Zalkin. *Arch. Biochem.* 80: 326; 333, 1959.
60. Brodie, A. F. *Fed. Proc.* 17: 196, 1958.
61. Brodie, A. F. *J. Biol. Chem.* 234: 398, 1959.
62. Snow, G. A. Resume des Communication, 2e Congres International de Biochimie, Paris, 95, 1952.
63. Noll, H. *J. Biol. Chem.* 232: 919, 1958.
64. Green, D. E., D. M. Ziegler and K. A. Doeg. *Arch. Biochem.* Nov. 1959.
65. Crane, F. L. and J. L. Glenn. *Biochim. et Biophys. acta* 24: 100, 1957.
66. Crane, F. L., J. L. Glenn and D. E. Green. *Biochim. et biophys. acta* 22: 475, 1956.
67. Criddle, R. S. and R. M. Bock. *Biochem. Biophys. Res. Communications* 1: 138, 1959.

AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

Symposium on Autonomic Pharmacology[1]

Chairman: MARK NICKERSON

NON-NICOTINIC GANGLION-STIMULATING SUBSTANCES

ULLRICH TRENDELENBURG

Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

MANY PHARMACOLOGISTS regard the terms 'stimulation of ganglion cells' and 'nicotine-like action' as synonymous. Such a view can only be accepted if it is proved that peripheral ganglion cells of the autonomic nervous system have only one type of receptor, namely the acetylcholine receptor with which acetylcholine, nicotine and nicotine-like substances react. To the author's knowledge no such evidence has been obtained. On the contrary, during recent years considerable evidence has accumulated indicating that non-nicotinic substances can stimulate ganglion cells of the peripheral autonomic nervous system.

This problem is of more than purely theoretical interest. Since ganglion-blocking substances are frequently used as pharmacological tools in the analysis of the mode of action of new substances, it is of great importance to determine whether all ganglion-blocking substances abolish the response of the ganglion cells to all stimulant agents. There is clear evidence that they do not. For example, competitive block by curarine (1) or by tetraethylammonium (TEA) (2) of the superior cervical ganglion of the cat does not abolish the ganglion-stimulating action of potassium chloride (KCl). It is thus possible to stimulate ganglion cells in the presence of fully effective amounts of so-called 'competitive ganglion-blocking substances' provided that the stimulant substance does not act on the acetylcholine receptors. The observation of Paton and Perry (3) that such 'competitive ganglion-blocking substances' do not change the resting potential of the ganglion cells provides further evidence for the view that during competitive block the ganglion cells themselves are excitable but that the acetylcholine receptors are blocked.

The nicotinic actions of acetylcholine have been defined by Dale (4) as actions which are *a*) not abolished by atropine, and *b*) abolished by an excess of nicotine. Since then this definition has been applied to all nicotine-like substances. Recent developments have shown that ganglionic blockade can be achieved either by depolarization of the ganglion cells or by competitive inhibition of the acetylcholine receptors. Both types of ganglionic blockade abolish the response of ganglion cells to nicotine-like substances. Non-nicotinic ganglion-stimulating substances, on the other hand, would then be expected to be ineffective during depolarization of ganglion cells (since depolarization renders ganglion cells inexcitable to all kinds of stimulation), whereas blockade of the acetylcholine receptors (by the so-called competitive ganglion-blocking substances) would not interfere with the action of non-nicotinic ganglion-stimulating substances. Such a working hypothesis is borne out by the following experimental observations.

Histamine, 5-hydroxytryptamine (5-HT) and pilocarpine are able to stimulate the superior cervical ganglion of the cat. This statement is based on the following evidence. *a*) Intravenous injections of histamine or pilocarpine into adrenalectomized spinal cats cause contractions of the nictitating membrane only when the superior cervical ganglion is intact; its removal

[1] Atlantic City, New Jersey, April 13, 1959.

abolishes this effect (5, 6). *b*) Intra-arterial injections of either of these two substances into the blood supply of the nictitating membrane fail to cause a contraction of the nictitating membrane, but injections into the blood supply of the ganglion (fig. 1) cause a response of the nictitating membrane. This response is restricted to the injected side and is abolished by section of the postganglionic fibers or by crushing the ganglion (6). Similar experiments with 5-HT have shown that this substance acts on both the smooth muscle of the nictitating membrane and the ganglion cells (7). *c*) Small amounts of either histamine, 5-HT or pilocarpine, potentiate the response of the nictitating membrane to submaximal preganglionic stimulation but not to postganglionic stimulation of equi-effective strength (8). *d*) These three substances increase the height of submaximal postganglionic action potentials elicited by weak preganglionic stimulation without affecting the height of the preganglionic action potentials (8,9). *e*) In the isolated and perfused superior cervical ganglion, 5-HT, histamine and pilocarpine potentiate the response of the nictitating membrane to injections into the perfusion fluid of nicotine-like substances, of KCl and to submaximal preganglionic stimulation; injected in larger amounts these three substances stimulate the isolated and perfused ganglion although stimulation is less regularly observed than in the non-perfused preparation (10–13). *f*) Histamine, 5-HT and pilocarpine are known to stimulate the adrenal medulla (6, 14–17).

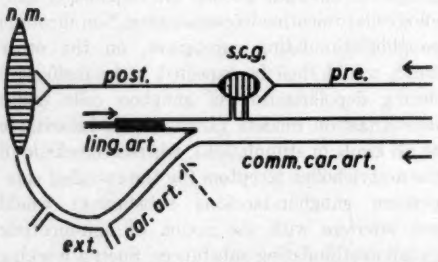


FIG. 1. Innervation and blood supply of the superior cervical ganglion (s.c.g.) and nictitating membrane (n.m.) of the cat. The central end of the tied and cut lingual artery is cannulated. Substances injected intra-arterially through this cannula are carried by the blood stream through the external carotid artery to the nictitating membrane without reaching the s.c.g. (injection to the nictitating membrane). After occlusion of the external carotid artery (at the broken arrow) the injected substance reaches the s.c.g. (injection to the ganglion), the stimulation of which is recorded as a contraction of the n.m.

Thus the evidence strongly suggests that histamine, 5-HT and pilocarpine are able to stimulate the superior cervical ganglion. The mode of action of these substances has been explored by the use of various pharmacological techniques. Depolarization of the ganglion cells by the injections of tetramethylammonium (TMA) abolished the response of the ganglion to these three substances without interfering with the direct action of 5-HT on the smooth muscle of the nictitating membrane (18). On the other hand, competitive block of the acetylcholine receptors by hexamethonium failed to abolish the response of the ganglion cells to histamine, 5-HT or pilocarpine (6, 7). The results obtained with nicotine were more complex. Paton and Perry (3) found that the depolarizing action of nicotine was of shorter duration than its ganglion-blocking action; in other words, some time after the administration of nicotine, ganglionic transmission was still blocked in spite of full repolarization of the ganglion cells. The authors concluded that the ganglion-block caused by nicotine consists of two phases: an initial phase of depolarization and a subsequent second phase of competitive block similar to that caused by hexamethonium. Without the use of electrophysiological methods the first phase can easily be differentiated from the second by the use of KCl: during depolarization (phase 1) the ganglion cells do not respond to KCl, but they do respond during competitive block (phase 2) (18). Similarly histamine, 5-HT and pilocarpine fail to stimulate the superior cervical ganglion when injected shortly after an initial injection of nicotine (phase 1, fig. 2b), while they are effective during prolonged administration of nicotine (phase 2, fig. 2d). This has been demonstrated both after intra-arterial (fig. 2) and intravenous injections (fig. 3) of ganglion-blocking amounts of nicotine (18).

The evidence so far discussed shows that histamine, 5-HT and pilocarpine, although capable of stimulating ganglion cells, are not nicotine-like but rather potassium-like. Since these three non-nicotinic ganglion-stimulating substances also have direct effects on vascular smooth muscle, it is conceivable that their ganglionic effects may be secondary to stimulation of vascular smooth muscle and to the liberation of potassium from such smooth muscle (19). This view, however, does not seem to be tenable, since

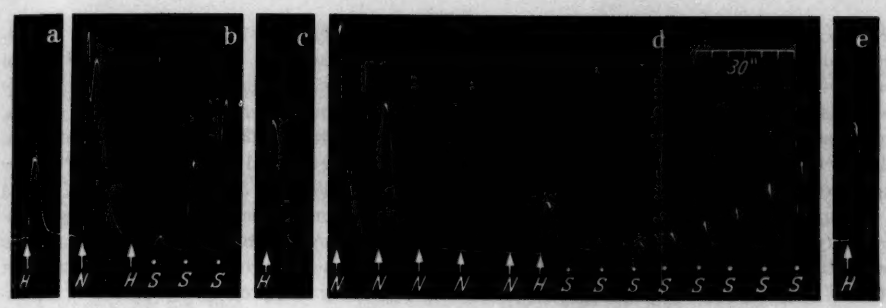


FIG. 2. Biphasic blocking action of nicotine. Cat under chloralose (80 mg/kg), normal nictitating membrane, preganglionic fibers cut. Intra-arterial injection of 5 μg histamine (H) and of 200 μg nicotine (N) to the ganglion (see fig. 1). Supramaximal preganglionic stimulation for 5 sec. (25 shocks/sec) at (S). Time interval between tracings 15 min. each. Note abolition of ganglionic action of histamine when histamine is injected shortly after nicotine (b) (first phase of nicotine block). The response to histamine is not abolished during prolonged block of the ganglion by nicotine (d) although block of transmission is much more pronounced (second phase of nicotine block).

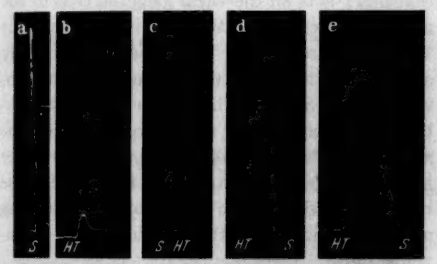


FIG. 3. Biphasic blocking action of nicotine. As in fig. 2, 1 mg/kg atropine i.v. before (a). Tracings of right (upper curve) and left (lower curve) nictitating membrane. Supramaximal preganglionic stimulation for 5 sec. at S. Intra-arterial injections to the ganglion (see fig. 1) of 10 μg 5-HT (HT), of 50 μg acetylcholine (A) and of 4 mg potassium chloride (K). (a) and (b) controls. Intravenous injection of 12 mg/kg nicotine immediately before (c) abolished the response to all types of ganglionic stimulation (first, depolarizing phase of nicotine block). 30 min. later a second intravenous injection of 12 mg/kg nicotine (immediately before d) caused full ganglion block but did not abolish the response to 5-HT and KCl (second, competitive phase of nicotine block). A similar result was obtained 30 min. later (e) immediately after a third intravenous injection of 24 mg/kg nicotine.

cocaine, morphine and methadone abolish the response of the superior cervical ganglion to histamine, 5-HT and pilocarpine without interfering with the direct vascular actions of these three substances (6, 7, 20, 21). These effects are obtained with amounts of cocaine, morphine and methadone which do not affect the response of the ganglion to preganglionic stimulation, to nicotine-like substances or to KCl (6, 20, 21).

Thus histamine, 5-HT and pilocarpine appear to act by combining with receptors which differ from the acetylcholine receptors of the ganglion cells. Some information is available on the specificity of these postulated receptors. The antihistaminic substance mepyramine (pyrilamine) abolishes the response of the ganglion to histamine without interfering with that to 5-HT or pilocarpine (6, 7). Atropine, on the other hand, abolishes selectively the response of the ganglion to pilocarpine (6, 7, 9, 10). This effect of atropine is highly specific and differs from the weak ganglion-blocking action of larger amounts of atropine (22). This curious effect of small amounts of atropine caused considerable controversy when Dale's definition of nicotine-like actions was applied to pilocarpine. In atropinized animals pilocarpine regularly failed to stimulate ganglion cells (23–25), whereas certain ganglion-stimulating properties were detected in non-atropinized animals or preparations (5, 6, 10, 26–28). The answer seems to be that pilocarpine stimulates ganglion cells by attaching itself to specific receptors which are highly susceptible to atropine.

The majority of the experiments discussed so

far was carried out on the superior cervical ganglion and on the adrenal medulla of the cat. Evidence from sympathetic ganglion cells of other species is not available, but further experiments suggest strongly that other sympathetic ganglion cells of the cat react similarly. Under certain experimental conditions intravenous injections of histamine or pilocarpine are known to cause a secondary rise of blood pressure. In the case of histamine this secondary rise is mainly due to stimulation of the adrenal medulla (14, 29, 30). But adrenal medullary stimulation plays only an insignificant role in the secondary rise of blood pressure after pilocarpine (6, 28), and this rise seems to be due to general stimulation of sympathetic ganglion cells. It is abolished by sympatholytics (28), by TM 10 (choline 2:6 xylyl ether bromide) (21), by cocaine (25, 30), by atropine (28) and during the first phase of the nicotine block (21); it is not abolished by hexamethonium (30), by TEA (28) and during the second phase of the nicotine block (21), (compare with table I).

There is little information available on non-nicotinic receptors in vagal ganglion cells. Histamine has no action on the intramural cholinergic ganglion cells of the guinea-pig ileum, whereas it stimulates those of the rabbit duodenum (31). Gaddum (32) postulated the presence of specific 'tryptamine receptors' for the guinea-pig ileum and later extended this concept to a two receptor theory according to which intestinal smooth muscle contains D-receptors (blocked by dibenzyline), whereas the nervous structures of the intestine contain M-receptors (blocked by morphine and methadone) (33). Rocha e Silva, Valle and Picarelli (34) reached a different conclusion. They found that the response of the guinea-pig ileum to 5-HT was abolished after a short exposure of the intestine to ganglion-stimulating amounts of nicotine (phase 1) or after cocaine; the response to 5-HT was not abolished by the prolonged exposure of the intestine to large amounts of nicotine (phase 2) or by hexamethonium. The authors concluded that 5-HT had no effect on ganglion cells but acted on the postganglionic nerve endings. Comparison with table I shows a remarkable parallelism of their results with those obtained on the superior cervical ganglion; this suggests strongly that 5-HT acts on the vagal ganglion cells of the myenteric plexus by attaching itself to specific 'tryptamine receptors.' Such a conclusion fully agrees with the view of Gaddum and his coworkers (32, 33, 35). The M-receptors of the intestine are equivalent to the receptors of the superior cervical ganglion; the D-receptors of the intestine seem to correspond to the 'tryptamine receptors' of the smooth muscle of the nictitating membrane, since the latter are blocked by dibenzyline (36).

TABLE I. SUMMARY OF RESULTS OBTAINED ON THE SUPERIOR CERVICAL GANGLION

	Nicotine-like Substances, Synaptic Transmission	Non-nicotinic Ganglion-Stimulating Substances	KCl
Tetramethyl-ammonium	−	−	−
Nicotine, first phase	−	−	−
Nicotine, second phase	−	+	+
Hexamethonium	−	+	+
Cocaine	+	−	+
Morphine and methadone	+	−	+
Atropine	+	Hist. +	+
		5-HT. +	
		Pilo. −	
Pyrilamine	+	Hist. −	+
		5-HT. +	
		Pilo. +	

Response inhibited: −; response unchanged: +.

SUMMARY

Evidence is presented for the view that histamine, 5-hydroxytryptamine and pilocarpine stimulate sympathetic ganglion cells of the cat by a mode of action different from that of acetylcholine and other nicotine-like substances. These agents are therefore described as 'non-nicotinic ganglion-stimulating substances.' Their effects are abolished by depolarization of the ganglion cells but not by competitive blockade of the acetylcholine receptors. These substances seem to attach themselves to specific receptors of the ganglion cells.

REFERENCES

1. BROWN, G. L. AND W. FELDBERG. *J. Physiol.* 86: 10 P, 1936.
2. ACHESON, G. H. AND S. A. PEREIRA. *J. Pharmacol.* 87: 273, 1946.
3. PATON, W. D. M. AND W. L. M. PERRY. *J. Physiol.* 119: 43, 1953.
4. DALE, H. H. *J. Pharmacol.* 6: 147, 1914.
5. DALE, H. H. AND P. P. LAIDLAW. *J. Physiol.* 45: 1, 1912.
6. TRENDELENBURG, U. *Brit. J. Pharmacol.* 9: 481, 1954.

7. TRENDELENBURG, U. *Brit. J. Pharmacol.* 11: 74, 1956.
8. TRENDELENBURG, U. *J. Physiol.* 135: 66, 1957.
9. MARRAZZI, A. S. *J. Pharmacol.* 65: 18, 1939.
10. AMBACHE, N. *J. Physiol.* 110: 164, 1949.
11. KONZETT, H. *J. Mt. Sinai Hosp. New York* 19: 149, 1952.
12. ROBERTSON, P. A. *J. Physiol.* 125: 37P, 1954.
13. TRENDELENBURG, U. *J. Physiol.* 132: 529, 1956.
14. BURN, J. H. AND H. H. DALE. *J. Physiol.* 61: 185, 1926.
15. FELDBERG, W., B. MINZ AND H. TSUDZIMURA. *J. Physiol.* 81: 286, 1934.
16. LECOMTE, J. *Arch. internat. pharmacodyn.* 100: 457, 1955.
17. SZCZYGIELSKI, K. *Arch. exper. Path. u. Pharmakol.* 166: 319, 1932.
18. TRENDELENBURG, U. *Arch. exper. Path. u. Pharmakol.* 230: 448, 1957.
19. MACMILLAN, W. H. AND J. R. VANE. *J. Pharmacol.* 118: 182, 1956.
20. TRENDELENBURG, U. *Brit. J. Pharmacol.* 12: 79, 1957.
21. TRENDELENBURG, U. Unpublished results.
22. KONZETT, H. AND E. ROTHLIN. *Helvet. physiol. et pharmacol. acta* 7: C46, 1949.
23. VON EULER, U. S. AND B. DOMEIJ. *Acta pharmacol. et toxicol.* 1: 263, 1945.
24. HEATON, T. B. AND M. H. MACKEITH. *J. Physiol* 63: 42, 1927.
25. KOPPANYI, T. *J. Pharmacol.* 65: 19, 1939.
26. BACQ, Z. M. AND A. SIMONART. *Arch. internat. pharmacodyn.* 60: 218, 1938.
27. HOET, J. *Ann. physiol. physicochim. biol.* 4: 714, 1928.
28. ROOT, M. *J. Pharmacol.* 101: 125, 1951.
29. SLATER, I. H. AND P. E. DRESEL. *J. Pharmacol.* 105: 101, 1952.
30. TRENDELENBURG, U. *J. Physiol.* 129: 337, 1955.
31. AMBACHE, N. AND A. W. LESSIN, *J. Physiol.* 127: 449, 1955.
32. GADDUM, J. H. *J. Physiol.* 119: 363, 1953.
33. GADDUM J. H. AND Z. P. PICARELLI. *Brit. J. Pharmacol.* 12: 323, 1957.
34. ROCHA E SILVA, M., J. R. VALLE AND Z. P. PICARELLI. *Brit. J. Pharmacol.* 8: 378, 1953.
35. GADDUM, J. H. AND K. A. HAMEED. *Brit. J. Pharmacol.* 9: 240, 1954.
36. THOMPSON, J. W. *J. Physiol.* 141: 46, 1958.

ENZYMIC OXIDATION OF PHARMACOLOGICALLY ACTIVE AMINES

PAUL HAGEN[1] AND NORMAN WEINER

Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

THE AIM of this paper is to present a brief resumé of the amine oxidases of mammalian tissues with emphasis on their differences and similarities particularly with regard to substrate and inhibitor specificities, differences in distribusion and, where known, differences in functions. Detailed accounts of other aspects of these enzymes have been presented elsewhere (1–3).

Amine oxidases carry out the following general reaction:

$$R{-}CH_2{-}NH_2 + O_2 + H_2O \longrightarrow R{-}C{-}HO + H_2O_2 + NH_3$$

The aldehyde may be further oxidized to the corresponding acid (4) by aldehyde oxidases of the tissues and the hydrogen peroxide decomposed by catalase (5). Differences in the nature of R and substitution on the α-carbon atom determine whether the compound will be a substrate of one or the other of the amine oxidases or indeed whether it will be oxidized at all.

The different amine oxidases differ in their distribution; they may be located in mitochondria or in the soluble components of the cytoplasm of solid organs or in the blood plasma or even lymph. They differ in their inhibitor specificity and, in fact, may be considered as belonging to one or two groups of amine oxidases depending on whether or not they are inhibited by carbonyl reagents (6).

The simplest classification of amine oxidases which fits with our present knowledge of these enzymes is shown below. (The characteristic features of each type will be considered in turn.) It is possible that with future study more types of amine oxidases may be discovered or that two or more of those listed may be shown to be identical.

[1] Present address: Dept. of Biochemistry, Univ. of Manitoba, Winnipeg, Canada.

CLASSIFICATION OF AMINE OXIDASES

Amine oxidase = Monoamine oxidase
Histaminase = Diamine oxidase
Mescaline oxidase
Spermine oxidase
Benzylamine oxidase

AMINE OXIDASE (MONOAMINE OXIDASE) (MO)

This enzyme deaminates amines of the general type R $-CH_2-NH_2$ where R may be an aryl or alkyl group with various substituents and where the amine group must be a primary amine, a methyl amine, an ethyl amine or dimethyl amine. Some of the better substrates in this group are n-hexylamine, isoamylamine, phenylethylamine, tyramine, and tryptamine. Certain long chain diamines, such as dodecamethylene diamine and octadecamethylene diamine (7) are also oxidized by amine oxidase (MO), as are some short chain diamines in which one amine group is poorly ionized.

Amine oxidase is widely distributed throughout mammalian tissues, having been detected in the mucosa of the alimentary canal, in the liver, kidney, and nervous tissue (see 1) in fairly large amounts. It is also present in lung, blood vessels, the adrenal gland, the heart of some species, the spleen, and probably in many other tissues. In some instances, it is not possible to state whether the enzyme is present in the characteristic cells of the tissue, or whether it is present only in the blood vessels and nerves which supply the tissue (see 1).

The enzyme is localized mainly or exclusively in mitochondria (8). The greatest difficulty has been encountered in trying to separate it from mitochondrial fragments and bring it into solution. It is because of this that most of our knowledge of the enzyme is based on its ability to oxidize various substrates and on its inactivation by various inhibitors.

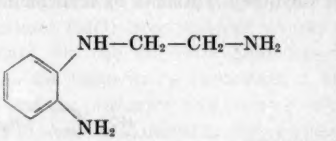
As already mentioned amine oxidase is an enzyme with a fairly broad substrate specificity. It oxidizes only aliphatic and aralkyl amines, aromatic amines such as aniline not being attacked by the enzyme. Primary amines are readily oxidized, as are secondary amines if one of the substituents is a methyl group. If larger groups are present, then the oxidation of the substrate is much reduced. Tertiary amines are poorly oxidized, and quaternary amines are not attacked by the enzyme.

Alpha-substituted amines are not oxidized by amine oxidase (9), and if this alpha-substituent is a methyl group the compound may competitively antagonize the oxidation of other amines (10). Amphetamine and ephedrine fall into this class of amine oxidase inhibitors. Beta-substituted amines may or may not be attacked by amine oxidase. If both available hydrogens are replaced by methyl groups, the compound no longer serves as a substrate for the enzyme (11). Monomethyl substitution, on the other hand, yields a compound which is still susceptible to amine oxidase. Beta-hydroxyl substitution results in more complex effects. In general, if the unsubstituted analogue is a good substrate, a beta-hydroxyl group impairs amine oxidase activity. Such is the case with dopamine and its beta-hydroxyl analogue, noradrenaline. If the unsubstituted compound is a poor substrate, beta-hydroxyl substitution improves the enzyme-substrate interaction. Phenylethylamine is poorly oxidized by human, rat, or mouse brain amine oxidase, and in each case, beta-phenylethanolamine is a somewhat better substrate. Conversely, phenylethylamine is well oxidized by rabbit brain amine oxidase, but the beta-hydroxyl analogue is less readily attacked (12). Steric factors apparently are not important in this influence of the beta-hydroxyl group, since d- and l-isomers of noradrenaline appear to be oxidized at approximately equal rates in each species.

Among phenylethylamine derivatives (the classical substrates studied) aromatic ring substitution to form phenolic structures yields compounds which are considerably better substrates for amine oxidases. Tyramine and the diphenolic dopamine (3-hydroxy tyramine) are generally the most readily oxidized compounds. Para-substituted compounds are more readily attacked than meta-substituted analogues, which are in turn better substrates than ortho-substituted compounds (11). Methylation of the hydroxyl group results in a compound which is a poorer substrate in the case of the para- and meta-hydroxyphenylethylamines, but a better substrate in the case of the ortho-compound (13). It is possible that in the case of the unsubstituted ortho-compound, chelation between the hydroxyl and amine group and a metal impairs the enzyme-substrate interaction, and methylation blocks this interaction, thus facilitating the enzyme attack of the substrate. Monomethylation of the meta-hydroxy group of the catecholamines, adrenaline and noradrenaline, does not appear to interfere with the enzyme-substrate interaction. Metanephrine and

normetanephrine are attacked at approximately the same rate as adrenaline and noradrenaline respectively (14).

A number of diamines are oxidized by amine oxidase (MO) (7, 15). In fact, a greater variety of diamines seem to be oxidized by amine oxidase (MO) than by histaminase (DO). The R in the general formula $R—CH_2NH_2$ may be a straight chain polymethylene group with an NH_2 at the other end, provided the alpha-amino group is separated from the omega-amino group by at least six carbon atoms; it is much more readily oxidized if the two amine groups are separated by ten or more methylene groups (7). The omega-nitrogen may be primary, secondary, tertiary, or even quaternary, without preventing the enzymic oxidation, which in any case only occurs at the opposite end of the molecule (16). Short chain diamines may also be oxidized by amine oxidase (MO) if one of the amine groups is a considerably weaker base than the other, so that the compound is essentially monocationic (15). Thus the following compound is a substrate of amine oxidase (MO):



The substrate specificity of amine oxidase is in large part dependent upon the source of the enzyme. There are considerable differences in the relative rates of oxidation of various amines in different tissues of the same species and in the same tissue of different species. In mouse, for example, the following organ variations have been observed. Mouse liver amine oxidase oxidizes tryptamine at a considerably faster rate than it oxidizes 5-hydroxytryptamine; in mouse kidney and in mouse brain tissue, on the other hand, 5-hydroxytryptamine is oxidized somewhat more readily than tryptamine. In mouse mastocytoma tissue tryptamine is readily oxidized, whereas 5-hydroxytryptamine is not attacked at a detectable rate (17). Phenylethylamine is readily oxidized by mouse liver and mastocytoma tissue, but is a poor substrate for mouse brain amine oxidase (14). Other examples of organ variations in the relative rates of oxidation of amines by amine oxidase have been reported (see 1).

Variations in substrate specificity of amine oxidase (MO) of the same tissue from different species are equally striking. Brain amine oxidase from several species has been systematically studied (12), and the following differences have been observed for several amines. Tryptamine and 5-hydroxytryptamine are poorly oxidized by brain amine oxidase from cat and human; they are better oxidized by rat and mouse brain (fig. 1). In the case of the latter two species, 5-hydroxytryptamine is a slightly better substrate than tryptamine. In rabbit and guinea pig, these two amines are also readily oxidized, but here, tryptamine is a better substrate than the 5-hydroxy analogue. Phenylethylamine is a poor substrate for brain amine oxidase in all of these species except rabbit; in this species, its rate of oxidation approaches that of tyramine (fig. 2).

The behavior of amine oxidase (MO) toward inhibitors appears more consistent than its behavior towards different substrates. This fact has led Blaschko to suggest that amine oxidases might be better classified according to their susceptibility toward inhibitors rather than their substrate specificity (6). Systematic studies of amine oxidase inhibitors, like systematic studies of substrate specificity have unfortunately been relatively few, and considerably more work along these lines should be done to broaden our understanding of these enzymes.

The amines with alpha-methyl substitution are competitive inhibitors of amine oxidase. The best known examples are ephedrine and amphetamine. Mann and Quastel (18) observed that amphetamine was a better inhibitor than ephedrine. The principal difference between these two compounds is the presence of a beta-hydroxyl group on the ephedrine molecule. It has been suggested that this hydroxyl group in some way lowers the affinity of the compound for the enzyme. In some preparations phenylethylamine is a better substrate than phenylethanolamine; again the suggestion has been made that the hydroxyl group lowers affinity, and so accounts for both these differences. However, in some tissues, phenylethanolamine is a better substrate than phenylethylamine (14). It is possible that in these tissues ephedrine might be a better inhibitor than amphetamine. This speculation has yet to be tested.

Certain monoamidines and diamidines are inhibitors of amine oxidase. Pentamidine and propamidine are powerful inhibitors (19). The inhibition by these compounds does not seem to be of a simple competitive nature.

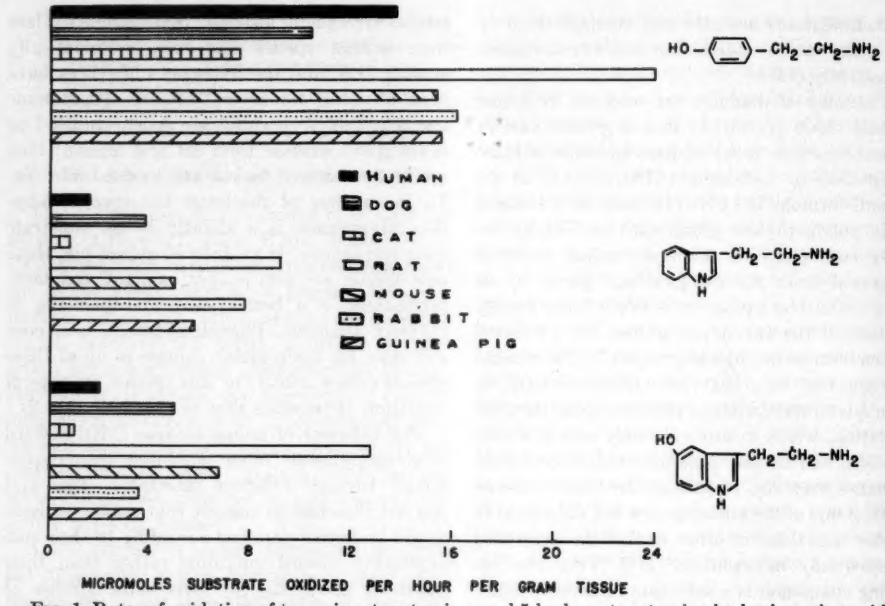


FIG. 1. Rates of oxidation of tyramine, tryptamine, and 5-hydroxytryptamine by brain amine oxidase of several mammalian species.

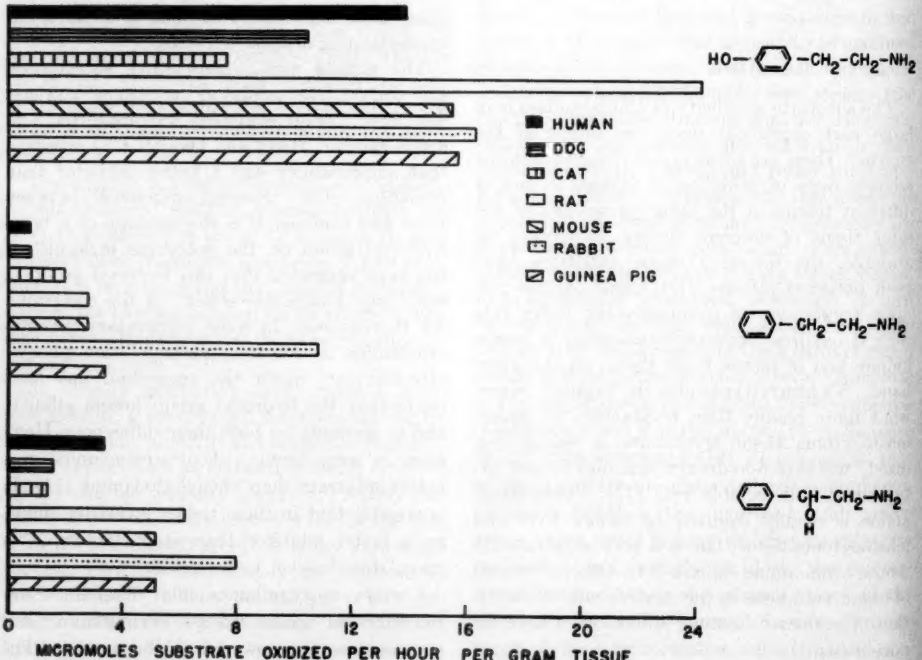


FIG. 2. Rates of oxidation of tyramine, phenylethylamine, and phenylethanolamine by brain amine oxidase of several mammalian species.

Octyl alcohol was one of the first inhibitors of amine oxidase discovered (20). Cocaine, nupercaine, and to a lesser extent other local anesthetics inhibit amine oxidase (21), as do some antihistiminic agents (22). No systematic study of the nature or specificity of the inhibition by these compounds has been reported.

A recently discovered group of amine oxidase inhibitors consists of certain hydrazine derivatives. Iproniazid is the classical example of these drugs (23). These compounds inhibit amine oxidase (MO) obtained from all sources at fairly low concentrations (10^{-4} M), and have been used for the *in vivo* inhibition of this enzyme (24). The inhibition can be partly antagonized if large amounts of substrate are added to the medium before or together with the iproniazid (25). However, once the inhibition is established, it is apparently irreversible.

Carbonyl reagents, such as semicarbazide and cyanide, do not inhibit amine oxidase (MO), and this is an important characteristic of the enzyme (1, 6).

One can infer from the data presented that amine oxidases (MO) from different sources are not identical, but exhibit subtle differences, depending on the tissue and species studied. It is probable that the primary active center to which the amine attaches is identical or very similar in the various tissues and species. However, it seems likely that adjacent groups on the protein molecule do differ, and these adjacent structures serve to reinforce or diminish the enzyme-substrate interaction, depending on the nature of the substrate. Iproniazid apparently acts at the primary site and inhibits the enzyme-substrate interaction of all amine oxidases (MO) and substrates tested. It seems possible that certain other inhibitors might differentially antagonize one or a few enzyme-substrate interactions and not others by interfering with the reinforcing or secondary forces. Selective amine oxidase (MO) inhibition might thus be possible. However, since species and tissue differences do exist, one must be cautious in applying the results of studies on amine oxidase from one species to another or from one tissue to another, without specific testing.

Biological Importance of Amine Oxidase (MO). There is still much uncertainty as to the real function or functions of amine oxidase. Animals seem to continue living without any obvious disturbances of function when their amine oxidase has been inactivated (with iproniazid), in complete contrast to the untoward effects produced by the *in vivo* inhibition of certain other enzymes, such as cholinesterase or cytochrome oxidase. The high activity of amine oxidase in the alimentary mucous membrane and in liver suggests that the enzyme may play or in the past may have played a role in the destruction of amines entering the body from ingested food or of amines formed by bacterial activity in the gut.

The other obvious function which has been ascribed to the enzyme is that of metabolizing amines produced in the animal body. In fact one of the first reports of the presence of the enzyme resulted from the search for a pathway of adrenaline metabolism (10). There is no doubt that adrenaline and noradrenaline can be metabolized by this enzyme in a number of tissues of a number of species. However, the rate of oxidation of these two catechol amines by this enzyme is very slow compared with the rates of oxidation of a number of other substrates. We know how that adrenaline and noradrenaline can be metabolized by ring methylation (26) and it is possible that they are also inactivated by being taken up into protein in exchange for ammonia (27). It should be emphasized that inactivation of a pharmacologically active compound is not necessarily the same as metabolism of the compound. Diffusion through body water or selective uptake by certain cells and tissues may so effectively remove a compound from sites at which it produces its pharmacological effects that a rapid rate of metabolism does not have to be postulated in the case of compounds the duration of whose effects is brief as in the case case of adrenaline and noradrenaline. Thus the fact that the effects of adrenaline and noradrenaline pass off rapidly and that they are metabolized very slowly by amine oxidase or other enzymes is not inconsistent provided one does not confuse inactivation and metabolism.

Within the past few years the discovery that the major catechol amine in nervous tissue is dopamine (28) has led to speculation as to whether it is present merely as a precursor of noradrenaline or whether it has a function in its own right. Dopamine is one of the best known substrates of amine oxidase. Two possible functions might be served by amine oxidase in the

destruction of dopamine; namely, the destruction of a biological agent with a hitherto unknonw function or the destruction of a precursor of a biological agent to prevent overproduction of its product noradrenaline (29). The same argument might be applied to another amine with biological activity, 5-hydroxytryptamine. So far this compound has no known function; however, as it is a moderately good substrate for amine oxidase (MO) in certain tissues (30) the enzyme might have some role in its metabolism or in regulating the amount available.

HISTAMINASE (DIAMINE OXIDASE, DO)

This enzyme was originally called histaminase, because it was first reported as the enzyme responsible for the inactivation of histamine in mammals (31). Later when it was realized that it oxidizes a number of aliphatic diamines more readily than histamine, the name diamine oxidase was suggested (32). Actually it has been known for some time that amine oxidase (MO) oxidizes a wider variety of diamines than histaminase (DO) and it is now known that histaminase (DO) also oxidizes certain monoamines (33). It would seem that the terms monoamine oxidase and diamine oxidase might be misleading, implying a specificity of action which these enzymes do not possess. One might facetiously suggest calling these enzymes pseudomonoamine oxidase and pseudodiamine oxidase.

Histaminase (DO) oxidizes a number of aliphatic diamines in which the two amine groups are separated by not more than ten methylene groups. Oxidation occurs only at one end of the molecule and the amine group where oxidation occurs must be a primary amine. In this respect histaminase (DO) differs from amine oxidase (MO). The second basic group need not be a primary amine, it may be a secondary or tertiary amine or even a guanidine, amidine or pyridine group (see 3). However, quaternization at the ω-N prevents the compound from being a substrate of the enzyme (16). The presence of the second nitrogen is not an absolute requirement. In some cases an oxygen atom serves quite well. Indeed pentane-5-ol-1-amine is oxidized at 58% of the rate of cadaverine (33), one of the best known substrates of histaminase (DO).

Histaminase is found in highest activity in the mucous membrane of the alimentary canal, in liver, kidney and lung (see 3). Its activity in blood is low except in the blood of women during pregnancy (34), where its concentration rises progressively from the end of the first trimester and falls to low levels within several days of delivery. The plasma histaminase apparently originates in the placenta, which has a highly active histaminase (34).

Histaminase is inhibited by carbonyl reagents such as aminoguanidine, semicarbazide, and cyanide. In this respect the enzyme differs from amine oxidase, which is not inhibited by these reagents.

Biological Functions of Histaminase (DO). As in the case of amine oxidase we do not know of any certain function of this enzyme. Again the high activity in alimentary mucosa and liver suggests that a major function may be, or may have been, to oxidize amines entering the body from the alimentary canal, for example, histamine, cadaverine, putrescine, and agmatine, which are known to be formed by decarboxylation of the corresponding amino acids by intestinal bacteria. Inhibition of histaminase *in vivo* by aminoguanidine does not seem to have harmful effects in the normal animal.

MESCALINE OXIDASE

The existence of this enzyme in rabbit liver was demonstrated twenty years ago (35). Very little is known about it apart from its ability to oxidize mescaline. It differs from amine oxidase (MO) in being sensitive to cyanide (35) and insensitive to octyl alcohol (20). Little is known about the ability of this enzyme to oxidize other substrates or about its intracellular location. It is possible that this may be the enzyme of rabbit liver mitochondria which oxidizes histamine. However, more studies are needed to establish this.

Apparently the possession of this enzyme confers on the rabbit its peculiar insensitivity to mescaline. Rabbits can tolerate without ill effects seventy times the dose of mescaline which is toxic to other animals (36). One can only postulate that the function of this enzyme in rabbits is to detoxicate mescaline, or some other amines in the diet or formed in the alimentary canal as products of the intense bacterial fermentation known to occur in the large intestine of the rabbit. This enzyme deserves considerably more study.

SPERMINE OXIDASE

Spermine oxidase was discovered in ruminant blood plasma about six years ago (37) and has since been partially purified (38). In addition to spermine and spermidine this enzyme oxidizes a number of monoamines but at a considerably slower rate (37). The preference for spermine and sperimidine is quite remarkable; mere alteration of the number of carbon atoms between the nitrogens not only makes the compound no longer a substrate but may even make it an inhibitor. The enzyme is inhibited by carbonyl reagents and by iproniazid (6).

This enzyme is found only in the plasma of ruminants. Its activity is very low in newborn ruminants and increases as the animal increases rumination, which parallels the development of bacterial fermentation in the rumen (39). It would seem logical to assume that this enzyme becomes activated in response to the absorption of spermine, a highly toxic material (40) which may be formed as a result of bacterial activity in the rumen, and that its function may be to detoxicate the spermine (37). This is in fact the only member of the amine oxidases to which we can assign a biologic role and feel that we are probably close to the truth.

BENZYLAMINE OXIDASE

This is found in the plasma of pig, horse, dog and some other mammals (6). It is not found in plasma of primates, marsupials, rabbits or cats. In addition to benzylamine a number of monoamines are also oxidized. Spermine and spermidine are only slowly oxidized, mescaline is oxidized quite rapidly (6). This enzyme is inhibited by carbonyl reagents and to a slight degree by iproniazid (6). In general this enzyme is very similar to spermine oxidase, the principal difference being that it occurs in the blood of different mammalian species and shows a different substrate specificity pattern. Any function ascribed to this enzyme would be merely speculative.

The function of each of these amine oxidases has been discussed briefly under each enzyme. It is clear that with the possible exception of spermine oxidase very little is known about their functions. The only function which may be tentatively ascribed to amine oxidases in general is that they may act to detoxicate amines entering the body from the alimentary canal. This assumption finds circumstantial support in the high concentration of these enzymes in the intestinal mucous membrane and liver and, in the case of spermine oxidase and benzylamine oxidase, in blood.

Although much has been made of the relation of the biosynthesis and metabolism of amines in brain to cerebral activity it is well to remember that our knowledge of the function of these amines particularly in mental phenomena is still so limited as to make it difficult at this time to implicate amine oxidases in these processes.

REFERENCES

1. Blaschko, H. *Pharmacol. Rev.* 4: 415, 1953.
2. Schayer, R. W. *Physiol. Rev.* 39: 116, 1959.
3. Zeller, E. A. In: *The Enzymes*, edited by J. B. Sumner and K. Myrbäck (vol. II, part 1). New York: Acad. Press, 1951, p. 536.
4. Bernheim, M. L. C. *J. Biol. Chem.* 93: 299, 1931.
5. Tabor, H. *J. Biol. Chem.* 188: 125, 1951.
6. Blaschko, H., P. J. Friedman, R. Hawes and K. Nilsson. *J. Physiol.* 145: 384, 1959.
7. Blaschko, H. and J. Hawkins. *Brit. J. Pharmacol.* 5: 625, 1950.
8. Hawkins, J. *Biochem. J.* 50: 577, 1952.
9. Blaschko, H., D. Richter and H. Schlossmann. *Biochem. J.* 31: 2187, 1937.
10. Blaschko, H., D. Richter and H. Schlossmann. *J. Physiol.* 90: 1, 1937.
11. Beyer, K. *J. Pharmacol. & Exper. Therap.* 79: 85, 1943.
12. Weiner, N. *Fed. Proc.* 18: 457, 1959.
13. Randall, L. O. *J. Pharmacol. & Exper. Therap.* 88: 216, 1946.
14. Weiner, N. Unpublished observations.
15. Blaschko, H., M. L. Chatterjee and J. M. Himms. *Brit. J. Pharmacol.* 10: 314, 1955.
16. Barlow, R. B., H. Blaschko, J. M. Himms and U. Trendelenburg. *Brit. J. Pharmacol.* 10: 116, 1955.
17. Hagen, P. *Fed. Proc.* 18: 399, 1959.
18. Mann, P. J. G. and J. H. Quastel. *Biochem. J.* 34: 414, 1940.
19. Blaschko, H. and R. Duthie. *Biochem. J.* 39: 347, 1945.
20. Blaschko, H. *J. Physiol.* 103: 13P, 1944.
21. Philpot, F. J. *J. Physiol.* 97: 301, 1940.
22. Tickner, A. *Brit. J. Pharmacol.* 6: 606, 1951.
23. Zeller, E. A. and J. Barsky. *Proc. Soc. Exper. Biol. & Med.* 81: 459, 1952.
24. Schayer, R. W., K. Y. T. Wu, R. L. Smiley and Y. Kobayashi. *J. Biol. Chem.* 210: 259, 1954.
25. Zeller, E. A., J. Barsky and E. R. Berman. *J. Biol. Chem.* 214: 267, 1955.
26. Axelrod, J. *Science* 126: 400, 1957.
27. Clarke, D. D., M. J. Mycek, A. Neidle and H. Waelsch. *Arch. Biochem.* 79: 338, 1959.

28. Schümann, H. J. *Arch. exper. Path. u. Pharmakol.* 227: 566, 1956.
29. Blaschko, H. *Pharmacol. Rev.* 6: 23, 1954.
30. Blaschko, H. *Biochem. J.* 52: X, 1952.
31. Best, C. H. and E. W. McHenry. *J. Physiol.* 70: 349, 1930.
32. Zeller, E. A. *Helv. chim. Acta* 21: 880, 1938.
33. Fouts, J. R., L. A. Blanksma, J. A. Carbon and E. A. Zeller. *J. Biol. Chem.* 225: 1025, 1957.
34. Ahlmark, A. *Acta physiol. scandinav.* 9, Suppl. 28, 1944.
35. Bernheim, F. and M. L. C. Bernheim. *J. Biol. Chem.* 123: 317, 1938.
36. Slotta, K. H. and J. Müller. *Hoppe-Seylers Z. physiol. Chem.* 238: 14, 1936.
37. Hirsch, J. G. *J. Exper. Med.* 97: 345, 1953.
38. Tabor, C. W., H. Tabor and S. M. Rosenthal. *J. Biol. Chem.* 208: 645, 1954.
39. Blaschko, H. and R. Hawes. *J. Physiol.* 145: 124, 1959.
40. Tabor, C. W. and S. M. Rosenthal. *J. Pharmacol. & Exper. Therap.* 116: 139, 1956.

AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

Symposium on Toxicology[1]

Chairman: HAROLD C. HODGE

INTRODUCTION

HAROLD C. HODGE

University of Rochester Medical Center, Rochester, New York

TOXICOLOGY, traditionally descriptive, is moving more and more into studies of mechanism. These studies draw on the knowledge of physiological and biochemical functions exactly as do mechanism studies of the therapeutic effects of drugs. For those seeking toxic effects, e.g. to develop superior economic poisons and military chemical agents, structure-activity relations point to the trials of new compounds. For those interested in health and safety, knowledge regarding the control, treatment and in some instances the prevention of poisoning, can be drawn from an understanding of mechanisms.

In this symposium, three instances of modern work on the mechanism of poisoning will be presented. Dr. J. D. Judah will describe his efforts to locate biochemical mechanisms in the mitochondria dislocated by carbon-tetrachloride. These biochemical changes are correlatable with structural changes since revealed by electron microscopy. Dr. J. Henry Wills will consider molecular interactions of toxic molecules (organic phosphates) and their antagonists with receptor sites whose characteristics are gradually being elucidated. Dr. Aser Rothstein's data illuminate active transport mechanisms in the cell membrane as contrasted with events and structures on the membrane or beneath the membrane. Dr. Rothstein's initial impetus came from work on the mechanism of uranium poisoning for the Manhattan Project. These three papers illustrate the give and take of mechanism studies in toxicology; in each case the picture of the normal mechanisms of metabolism, molecular interaction and transport in the cell has been brought into sharper focus.

[1] Atlantic City, New Jersey, April 14, 1959.

MECHANISM OF ACTION OF CARBON TETRACHLORIDE

J. D. JUDAH[1] AND K. R. REES

Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania; University College Hospital Medical School and University College, London, England

IN PREVIOUS WORK (1) we put forward the theory that carbon tetrachloride acted upon liver cells by penetrating the membrane and attacking the mitochondrial substance. We believed that this brought about a progressive disruption of mitochondrial function which ended in the death of the cell.

Let me summarize the evidence on which this

[1] Present address: The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

view was based. Slices cut from the livers of rats poisoned with CCl_4 showed a marked reduction in oxygen uptake at 18 hours after administration. Examination of homogenates of such livers revealed that various oxidations were lower than those of control livers. For example, the oxidation of octanoate was abolished, that of pyruvate was reduced by 50% while citrate oxidation was inhibited by some 70%. The oxidation of α-ketoglutarate was unaffected, as was that of succinate, and there were small changes in the oxidation of choline, β-hydroxybutyrate and L-glutamate. Oxidative phosphorylation with glutamate as substrate was apparently intact.

Examination of mitochondria isolated from the livers of poisoned rats showed a series of changes which were reproducible and which pointed to membrane injury. Thus, spectrophotometric study revealed that the reduction of diphosphopyridine nucleotide, which proceeds extremely slowly in mitochondria isolated from normal rat liver, was much increased in preparations from poisoned rats; conversely, if the mitochondria were aged, the oxidation of pyridine nucleotide dependent substrates was greatly reduced, and could only be restored by addition of DPN (fig. 1). The aging effects were observed at different times with different substrates. Thus, the oxidation of citrate was affected within 5–10 hours after poisoning, the time of aging necessary being some 30 minutes. At this time, malic oxidation proceeded unimpaired, but aging for 120 minutes brought about considerable loss of activity. The oxidation of glutamate was normal. But at 18 hours after poisoning and some 60 minutes of aging, this also went down (fig. 2). All these reactions could be restored by addition of DPN, and we assumed that the mitochondria were both more accessible to added DPN and lost more readily their endogenous nucleotide.

We also studied the two-step oxidation of citrate to succinate, in presence of malonate. In mitochondria from the livers of CCl_4 poisoned rats, it was found that considerable amounts of α-ketoglutarate accumulated in the reaction vessels, amounting to some 50% of the citrate disappearing, whereas in the controls only small amounts of the keto-acid were found. Since simultaneous experiments showed that α-ketoglutarate oxidation was unimpaired when it was added as substrate, we inferred that the keto-acid produced by the oxidation of citrate was diffusing away from the enzyme system and that the subsequent step was slow until some fair quantity of α-ketoglutarate had built up, representing substrate levels in the reaction mixture. This suggests that some gross damage has been done to the mitochondrial structure, a hypothesis which is in keeping with the results already described.

This simple mechanism has been attacked on the grounds that the changes are unimportant, secondary consequences and that they merely re-

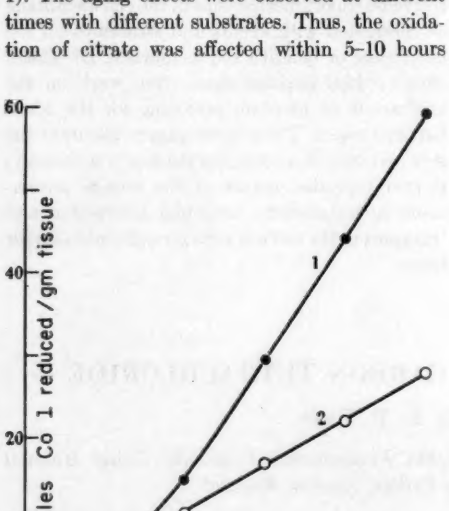


FIG. 1. Reduction of DPN in rat liver. Curve 1: CCl_4-poisoned rat liver. Curve 2: normal rat liver.

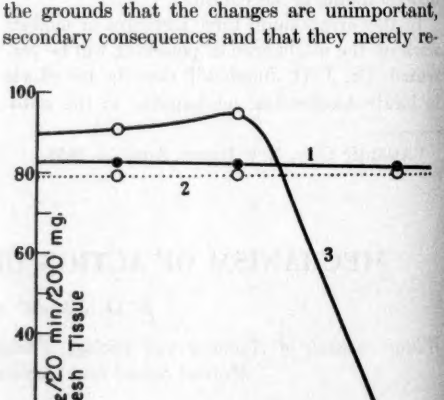


FIG. 2. Oxidation of L-glutamate in liver of CCl_4-poisoned rats. Curve 1: control; curve 2: 10 hr. after poisoning; curve 3: 18 hr. after poisoning.

flect the death of the liver cells. However, the earliest of the alterations observed (at 5–10 hours) is already extensive, since in order to pick up any change at all many cells must be affected. Furthermore, there was a steady sequence of events, different enzymes being affected at different times, and at least one of these enzymes, α-ketoglutarate oxidase, is complex and would not be expected to survive in dead cells; in fact it was found working at normal rates at 18–20 hours after poisoning. Furthermore, oxidative phosphorylation remained intact, and also the mitochondria were capable of synthesizing phosphatidic acid at this time, when an oxidizable substrate and ^{32}P were present in the medium. This is all in marked contrast to the findings in autolyzing livers (2, 3) where the loss of the respiratory function and of oxidative phosphorylation proceeds hand in hand, and survival may be measured in tens of minutes rather than in tens of hours.

Another possibility, suggested to me by Dr. E. P. Kennedy, was that the synthesis of lecithin was impaired directly by CCl_4, which he had found *in vitro* to be inhibitory to the enzyme which brought about the reaction of a diglyceride and cytidine diphosphate-choline. If this were so, all our findings and also the accumulation of neutral fat in liver cells after CCl_4 poisoning would be explained. On this basis, it would be hard to explain the well-known protective effect of choline, but not altogether impossible.

We had already found that phosphatidic acid synthesis proceeded perfectly well, and actually at more rapid rates in mitochondria from poisoned livers than in controls, an observation which has been repeated recently. This is in keeping with Kennedy's hypothesis. However, we extended our observations to intact animals (Judah and Rees, to be published). In these experiments, we dosed rats with CCl_4 by stomach tube, and 3 hours later injected a dose of ^{32}P (10 μC/kg); 2 hours after this, the animals were killed. The mixed lipids of the liver were extracted and counted and it was found that the specific activity was about 1.5 times the control level in the poisoned livers (table 1). Since the procedure employed would extract phosphatidic acid, the extract was passed through an ion-exchange column, and it was found that nearly all the activity of both control and treated livers was present in the neutral fraction which passed through the column. This eliminated the possibility that phosphatidic acid was accumulating. It is of interest that mitochondria isolated from these livers are also capable of synthesizing phosphatidic acid at greatly increased rates, e.g. at 1.7 times the control rates.

Studies of the blood levels of inorganic phosphate and its specific activity were carried out in order to rule out the chance that an increased specific activity of the inorganic phosphate was re-

TABLE 1. SPECIFIC ACTIVITY OF LIVER PHOSPHOLIPIDS IN CONTROL AND CCl_4-POISONED RATS

Animal	Fraction	Total Counts in Phospholipid	Specific Activity c.p.m./μg P
Control	whole liver	8700	16.0
	mitochondria	3850	15.9
CCl_4 (3–5 hr.)	whole liver	16600	31.4
	mitochondria	6700	31.0

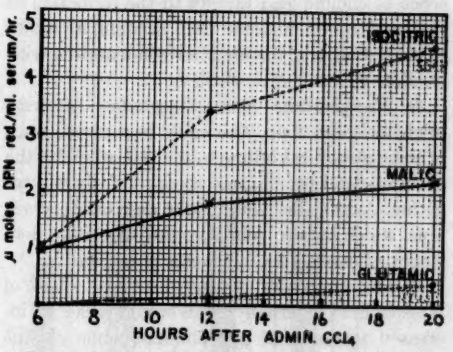


FIG. 3. Levels of isocitric, malic and glutamic dehydrogenases in the serum as a function of the time after administration of CCl_4 to rats.

TABLE 2. DISTRIBUTION OF MALIC DEHYDROGENASE IN CELLULAR FRACTIONS OF LIVER FROM CONTROL AND CCl_4-POISONED RATS*

Fraction	μ moles Co I reduced/gm wet wt. liver/h†	
	Control‡	CCl_4‡
Homogenate	615 (100)	280 (100)
Nuclei	14 (2)	7 (2.5)
Mitochondria	61.5 (10)	106 (38)
Supernatant	560 (91)	168 (60)

* System: phosphate buffer, pH 7.8, 0.033 M; KCl, 0.025 M; semicarbazide, 0.17 M; $MgSO_4$, 0.0067 M; CoI, 0.001 M; sodium L-malate, 0.05 M; NaCN, 2×10^{-4} M; temp. 20°C.

† Determined by Beckman D.U. spectrophotometer at 340 mμ.

‡ Numbers in parentheses represent percentage recovery of malic dehydrogenase activity.

sponsible for this result. We also studied the distribution of radioactive tracer in the phosphate of lecithin and cephalin, and found that it was normal. It would appear, therefore, that the proposed mechanism by which CCl_4 inhibits lecithin synthesis cannot be correct.

How do these results fit in with the observed accumulation of neutral fat and the protective effect of choline? They fit in well, and the following examples show how it is possible to explain them:

1) Phosphatidic acid synthesis may increase (as we found in our *in vitro* work), although it is hard to see how or why this should be, but in consequence the liver may rapidly be depleted of its choline by increased lecithin synthesis. This would account for protective effects by choline. The increased phosphatidic acid would in the absence of choline lead rapidly to the formation of diglyceride, which would then accumulate either as such or as triglyceride after condensation with another molecule of acyl-Co A.

2) Phosphatidic acid phosphatase activity might be speeded up. This could be a consequence of some structural injury to the cell, whereby the substrate and the enzyme come more readily together. Again, this would make diglyceride available, which in turn could produce a relative deficiency of choline and an excess of neutral fat.

3) Structural cell damage leading to loss of phospholipid externally: This would mean an increased turnover of phospholipid while choline or CDP-choline was available. After that, neutral fat would accumulate.

Finally, we have the results of some experiments which help to throw some light on the sequence of events in CCl_4 poisoning. Dr. K. P. Sinha, working under the direction of Dr. Rees, has studied the blood levels of isocitric, malic and glutamic dehydrogenases. At the same time, he has determined the intracellular levels of these enzymes in the cell fractions. Now, isocitric dehydrogenase has a large representation in the soluble fraction of the liver homogenate, though there is a fair amount (about 20%) in the mitochondria; malic dehydrogenase is present to about 60% in the supernate and 40% in the mitochondria, while glutamic dehydrogenase is purely mitochondrial (4). Sinha finds that 5–6 hours after poisoning, the levels of isocitric and malic dehydrogenases are markedly elevated in the blood, the former being higher (fig. 3). Glutamic activity is very low at this time. At 14 hours glutamic activity is still very low, but further increases are found in the other two systems. At 24 hours glutamic dehydrogenase makes its first appearance in any quantity.

The liver levels are as might be expected. Early in the course of poisoning there are losses in the soluble fractions of isocitric and malic dehydrogenases; the mitochondrial levels being intact (table 2). At 14–18 hours the mitochondrial activity of these enzymes begins to fall, but glutamic dehydrogenase activity is normal until 24 hours, at which point losses are observed.

We interpret this to mean that a change occurs early in the course of poisoning which permits the soluble cytoplasmic components to pass out of the cell and into the blood. This change correlates well in time with the observed changes in phospholipid turnover. We have studied calcium accumulation in poisoned livers, and find that this is insignificant until 18–24 hours have elapsed. There was no increase at 14 hours but a very high level at 24 hours (1.0 mg/gm wet weight). This means that although intracellular components are passing the cell-membrane, the cells can still maintain themselves and cannot be regarded as dead. It might be remarked that the mitochondrial changes observed in the older investigation were very well marked by 14 hours.

We should also like to point out that the loss of the glutamic dehydrogenase only becomes significant at a time when necrosis is well established and mitochondrial dissolution must be taking place.

We will continue our studies of this problem, and feel that there is still considerable room both for discussion and for experiment.

REFERENCES

1. Christie, G. S. and J. D. Judah. *Proc. Roy. Soc. Londen. ser. B.* 142: 241, 1956.
2. Gallagher, C. H., J. D. Judah and K. R. Rees. *J. Path. & Bact.* 72: 247, 1956.
3. Dawkins, M. J. R., J. D. Judah and K. R. Rees. *J. Path. & Bact.* 77: 257, 1959.
4. Christie, G. S. and J. D. Judah. *Proc. Roy. Soc. London. ser. B.* 141: 420, 1953.

DISCUSSION

THEODORE M. BRODY

Department of Pharmacology, University of Michigan, Ann Arbor, Michigan

I WOULD LIKE to take this opportunity to thank Dr. Hodge for allowing me to present some of our studies here. This is essentially a summary of papers which will be presented at this meeting (1, 2). I should also mention that our data are not in disagreement with that of Dr. Judah and his co-workers but are oriented toward the proposition that the changes seen in liver mitochondria from carbon-tetrachloride treated rats are indirect and may result from an alteration in liver flow and an anoxia rather than a direct effect of CCl_4 per se. We propose that the sympathetic nervous system and its neurohumors are involved, and to test this hypothesis have employed the use of adrenergic blocking agents phenoxybenzamine and ergotamine; pretreatment with reserpine; adrenalectomy and transection of the spinal cord. The test systems employed as criteria for indicating the changes induced by CCl_4 are *a*) measurement of oxidative phosphorylation, *b*) measurement of the activation of the magnesium-dependent ATPase, *c*) measurement of total liver lipid and *d*) determination of catechol amine levels in the adrenal glands as an index of the ability of CCl_4 to release these neurohumoral agents in the peripheral nervous system.

All treated rats received 2.5 ml/kg of CCl_4 orally 20 hours before sacrifice. Phenoxybenzamine (5 mg/kg subcutaneously) was administered simultaneously with CCl_4 administration and ergotamine (3 mg/kg subcutaneously) 2 hours prior to the CCl_4. Figure 1 shows the protective effect of these adrenergic blocking agents on the typical CCl_4 depression of liver mitochondrial oxidative phosphorylation. Section of the spinal cord at the level of T2 or T3 prevented almost completely the effects on liver mitochondria usually seen with CCl_4. These data are depicted in figure 2. The values in the right-hand panel are about 80–90% of those obtained with a cordotomized, control animal. Oxidative and phosphorylative values of mitochondria of cordotomized controls do not differ significantly from non-operated animals.

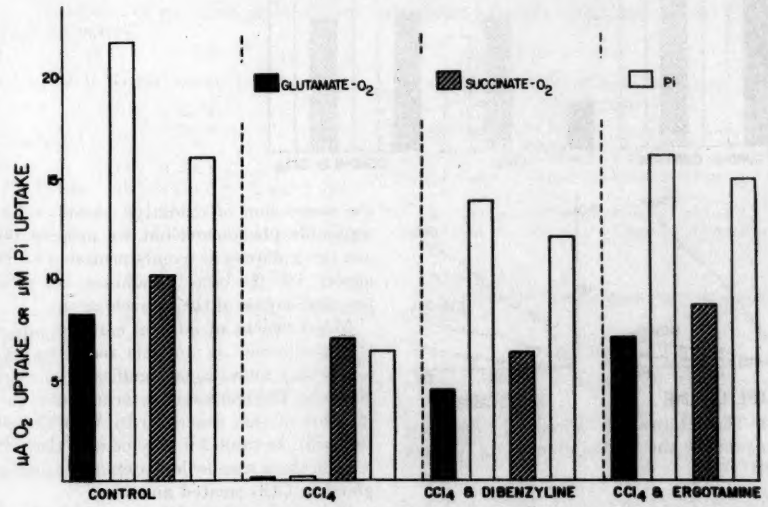


FIG. 1. The effect of phenoxybenzamine (Dibenzyline) and ergotamine in protecting against the action of CCl_4 on oxidative phosphorylation.

Rechnagel and Anthony (3) first demonstrated that CCl_4 transformed the 2,4-DNP-activated liver mitochondrial ATPase to a Mg activated one. Figure 3 shows the failure of CCl_4 to activate the Mg ATPase after treatment with the adrenergic blocking agents phenoxybenzamine and ergotamine. Generally, it was found that the degree of ATPase activation paralleled the extent of mitochondrial damage as measured by oxidative phos-

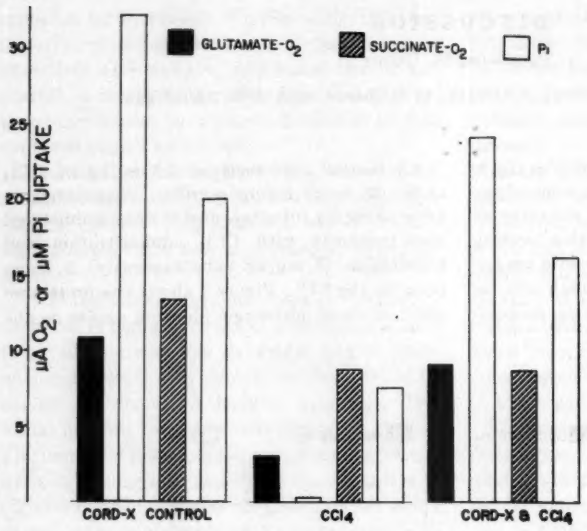


FIG. 2. The protective effect of cordotomy against CCl_4 depression of oxidative phosphorylation.

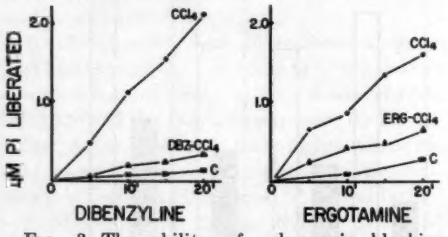


FIG. 3. The ability of adrenergic blocking agents to prevent the CCl_4-induced Mg ATPase activation.

phorylation. In figure 4 are shown the effects of reserpine pretreatment, adrenalectomy and spinal cord transection on the ATPase transformation. Both reserpine and adrenalectomy reduced by about 50% the activation of the mitochondrial Mg ATPase and there was no apparent activation if the spinal cord was previously transected.

A characteristic of CCl_4 intoxication is the marked fatty infiltration of the liver parechymal cells. That this is also an indirect effect is indicated by the data in table 1. These represent lipid levels determined by the method of Folch *et al.* (4) on samples of liver from which mitochondria were isolated for oxidative phosphorylation and ATPase studies. It can be seen that CCl_4 increased the total lipid from about 6% to 14% in 20 hours. The adrenergic blocking agents significantly lowered the total lipid accumulation. Adrenalectomy and cord section also protected against the CCl_4-induced accumulation of fat in the liver. Many investigators have felt that the lipid deposition and the depression of oxidative phosphorylation are separable phenomena but we propose that both can be explained as events mediated by the same agents, i.e. the catechol amines, but differing in the time course of their development.

Measurement of adrenal catechol amine levels was performed on animals receiving CCl_4, and levels were found to be significantly lowered after 20 hours. The method of determination was a modification of that described by Weil-Malherbe and Bone (5). In table 2 it may be seen that after cord section there was no depletion of the amines in the gland in CCl_4-treated animals.

We propose that CCl_4 exerts an effect on the central nervous system resulting in a massive sympathetic discharge. The latter has a two-fold effect. It causes a constriction of blood vessels supplying the liver with a resultant decrease in blood flow and a consequent anoxia. This produces the characteristic centrilobular necrosis and the metabolic changes described above. The second effect is one of stimulation of the adrenal medulla with a release of epinephrine and norepinephrine into the circulation which enhances the vascular response. Moreover, the sympathetic discharge increases the blood levels of unesterified fatty acids. That the catechol amines will trigger the release of UFA from fat depots has been demonstrated by Gordon and Cherkes (6) and White and Engel (7).

Section of the spinal cord should abolish these responses, which is in agreement with our experimental data. It is not possible to distinguish either by gross observations, histologic examination or

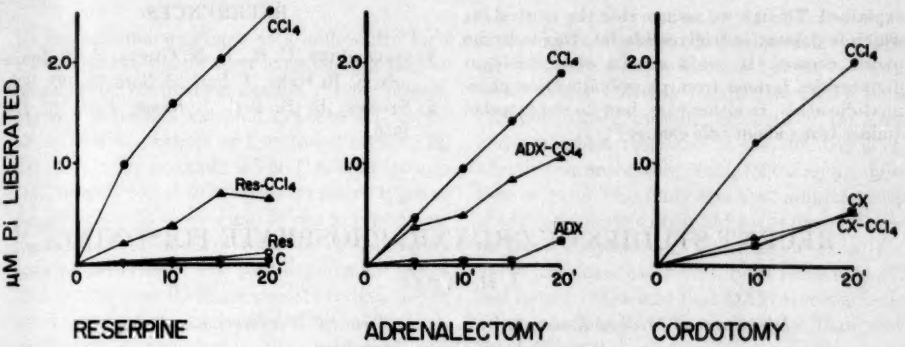


FIG. 4. The effects of reserpine pretreatment, adrenalectomy and cordotomy on the CCl_4-induced Mg ATPase activation.

TABLE 1. TOTAL LIVER LIPIDS

Group	Treatment	N	% wet wt. mean ± S. D.	p
1	None	14	6.3±1.2	
2	CCl_4 20 hrs.	25	14.0±2.6	1 vs. 2 .001
3	Adren. block.	3	6.5±0.5	
4	CCl_4 adren. block.	8	11.4±1.7	4 vs. 2 .01
5	Adrenalectomy	4	5.0±0.2	5 vs. 1 .01
6	CCl_4 after adrenalectomy	4	6.6±0.9	6 vs. 2 .001
7	Cord. section	6	5.3±0.4	7 vs. 1 .02
8	CCl_4 after cord. section	4	5.6±0.4	8 vs. 2 .001

N = number of animals.

by measurement of metabolic changes those livers of CCl_4-treated animals after cord section from normal, untreated controls. We believe therefore that stimulation of the central nervous system and a consequent sympathetic discharge is primarily responsible for the hepatotoxicity of CCl_4.

TABLE 2. ADRENAL CATECHOL AMINES (μg/adrenal pair)

Treatment	Epi	Norepi	Total	N	p
1. Control	33.9±5.3*	6.5±3.5	40.4±6.2	13	
2. CCl_4 20 hr.	21.3±7.0	6.2±2.5	28.3±5.5	12	1 vs. 2 .001
3. Cord section	36.6±5.3	9.7±2.9	46.1±4.7	6	
4. Cord sect. + CCl_4	40.4±5.0	9.2±4.2	49.6±4.8	6	2 vs. 4 .001

* Mean ± S.D.

N = number of determinations, each representing pooled adrenals from 2 rats.

REFERENCES

1. CALVERT, D. N. AND T. M. BRODY. *Fed. Proc.* 18: 375, 1959.
2. MOORE, K. E. AND T. M. BRODY. *Fed. Proc.* 18: 424, 1959.
3. RECHNAGEL, R. O. AND D. D. ANTHONY. *Fed. Proc.* 16: 105, 1957.
4. FOLCH, J., M. LEES. AND G. H. SLOANE-STANLEY. *J. Biol. Chem.* 226: 497, 1957.
5. WEIL-MALHERBE, H. AND A. D. BONE. *Biochem. J.* 51: 311, 1952.
6. GORDON, R. S., JR. AND A. CHERKES. *Proc. Soc. Exper. Biol. & Med.* 97: 150, 1958.
7. WHITE, J. E. AND F. L. ENGEL. *Proc. Soc. Exper. Biol. & Med.* 99: 375, 1958.

REPLY TO DISCUSSION

J. D. JUDAH

HAVING HEARD Dr. Brody's paper with interest, I would like to make the following points:

We have performed experiments in which adrenalectomy protected the liver to some extent against another hepatotoxic agent, thioacetamide. In this case, anti-histamine compounds also afforded almost complete protection. Nevertheless, we cannot draw the conclusion that either adrenaline (or other catechol amines) or histamine are in any way responsible for the action of thioacetamide.

It is hard to see how the catechol amines can affect the liver other than by vascular effects; Stoner (2) has failed to observe such effects after CCl_4 poisoning.

It is also hard to see how the fatty liver can be

explained. Though we assume that the neutral fat which is present is triglyceride fat, this is by no means certain. It could consist of alpha-beta-diglycerides formed from phospholipids or phosphatidic acids. In either case, how do the catechol amines bring about this change?

REFERENCES

1. Gallagher, C. H., D. N. Gupta, J. D. Judah and K. R. Rees. *J. Path. & Bact.* 72: 193, 1956.
2. Stoner, H. B. *Brit. J. Exper. Path.* 37: 176, 1956.

RECENT STUDIES OF ORGANIC PHOSPHATE POISONING

J. H. Wills

Directorate of Medical Research, U. S. Army Chemical Warfare Laboratories, Army Chemical Center, Maryland

My presentation will summarize some recent studies of antagonism of the toxic effects of P-containing anticholinesterases rather than those of the toxicities of these compounds. That P-containing esters inhibit cholinesterase has been known since the late 1930's and has been in print since 1943, when Hottinger and Bloch (1) reported that triortho-cresyl phosphate is an inhibitor of cholinesterase. Upon recognition of inhibition of cholinesterase as the major mechanism of acute toxicity of P-containing esters, it was apparent that atropine and other anticholinergic drugs would serve as antagonists to some of the lethal actions of those esters. Anticholinergic drugs antagonize symptoms of intoxication rather than the intoxication itself; furthermore, most of them do not reduce such nicotinic effects of the P-containing esters as the neuromuscular block that leads to weakness of the respiratory musculature, and finally to respiratory paralysis, even in atropinized animals. For these reasons, compounds have been sought to antagonize the basic mechanism of toxicity of the P-containing esters and, particularly, to combat neuromuscular block.

In the early days of research on the mechanism of action of the P-containing anticholinesterases, these compounds were considered to inhibit cholinesterase irreversibly at so rapid a rate that interference with the reaction seemed impossible. The first break in this apparently impenetrable front appeared in 1948, when Berry and Todrick (2) reported that pyrogallol, catechol, DOPA, adrenaline and hydroquinone are able to protect cholinesterase *in vitro* from inhibition by the potent cholinesterase inhibitor sarin (isopropyl methylphosphonofluoridate). There are three possible mechanisms for this protective activity: first, prevent phosphorylation of cholinesterase by competitive occupation of the active site of the enzyme molecule; second, destroy the phosphorylating compound before it has an opportunity to react with cholinesterase; and third, reactivate the inhibited cholinesterase. The first two of these mechanisms have potential value in preventing intoxication by anticholinesterase compounds and the third has the possibility of overcoming intoxication after its establishment.

Three types of drugs have been found to be active in preventing phosphorylation of cholinesterase by competitive union with that enzyme. These are: *1*) reversible cholinesterase inhibitors, such as physostigmine and prostigmine (3, 4); *2*) choline esters, such as acetylcholine and mecholyl (5–7); and *3*) local anesthetics, such as procaine and tetracaine (8, 9). Members of these three groups of drugs have significant prophylactic activity against poisoning by P-containing anticholinesterases in experimental animals; the reversible cholinesterase inhibitors have no therapeutic value, increasing the severity of poisoning when given after a P-ester, but the choline esters and the local anesthetics do have some therapeutic value. This latter type of activity by the choline esters depends probably upon their ability to displace the phosphorylating moiety from inhibited cholinesterase and to reactivate the enzyme (10). Therapeutic activity by local anesthetics may be related to the ability of these compounds to reduce release of acetylcholine by nerve endings (11), leading thereby to gradual decreases in both local and circulating levels of acetylcholine.

The first compounds found to destroy P-con-

taining anticholinesterases at a sufficiently high rate *in vitro* to justify their experimental trial as prophylactic compounds against P-ester poisoning were pyrogallol, catechol and catechol-derivatives, DOPA, phenols and hydroxyl amine (12, 13). Later, hydroxamic acids (14) and oximes (15, 16) were found to be the most active types of compounds both *in vitro* and *in vivo* in preventing inactivation of cholinesterase by P-containing anticholinesterases. The tertiary oxime DAM (2,3-butanedione 2-oxime) appears to be a better agent for preventing poisoning of the rat by anticholinesterase compounds than atropine and several other oximes (table 1). The apparent ineffectiveness of atropine is due to the fact that its action to prevent bronchoconstriction after inhalation of sarin vapor results in greater inhalation and absorption from the respiratory tract, of the lethal material, than occurs in animals given no atropine before being placed in the toxic atmosphere.

Table 2 shows that DAM affords some protection to the rat when 200 mg/kg are given intraperitoneally at times less than 4–5 hours before exposure to sarin vapor and considerable protection when this dose is given no more than 2–3 hours before exposure. A smaller dose of DAM gives equally good protection but for a shorter time. Askew (18) has found that DAM is less effective in the guinea pig, the monkey, the mouse and the rabbit than it is in the rat, but is still effective in preventing death following s.c. injection of sarin. She finds also that administration of atropine along with DAM gives more effective protection against s.c. injection of sarin than use of the oxime alone (19). Both Koon *et al.* (17) and Askew (19) found that DAM is less effective when administered therapeutically than when given prophylactically.

TABLE 1. EFFECTIVENESS OF ATROPINE AND VARIOUS OXIMES*

Compound	Dose Mg/Kg	Mean Survival Time (min.)
None		5
Atropine	10	4
MINA (pyruvaldehyde aldoxime)	25	6
2-PAM (2-formyl-1-methylpyridinium iodide oxime)	75	8
2,3-pentanedione 3-oxime	100	38
DAM (2,3-butanedione 2-oxime)	200	123

* Injected i.p. into rats 15 minutes before the initiation of exposure to sarin vapor (150 mg/cu. m.), in prolonging the mean survival time in the toxic atmosphere (17).

TABLE 2. EFFECTIVENESS OF DOSES OF DAM (2,3-BUTANEDIONE 2-OXIME)*

Injection Time	Dose	Mortality
−0.25 hr.	50	0/5
−1 hr.	50	2/5
−2 hr.	200	0/5
−3 hr.	200	1/5
−4 hr.	200	1/5
−5 hr.	200	5/5
None	0	5/5

* Given to rats i.p. at various times before initiation of exposure for 4 min. to sarin vapor (146 mg/cu. m.), in preventing mortality (17).

The original idea that the P-esters are irreversible inhibitors of cholinesterase seemed to render impossible any specific therapy for intoxication by the former compounds. Wilson (20) and Hobiger (21) first showed in 1951 that phosphorylated cholinesterase can be dephosphorylated and reactivated, slowly by water and more rapidly by hydroxyl amine and choline. Later, Wilson and Meislich (22) found nicotinehydroxamic acid methiodide to be a highly effective reactivator. Wilson reported in 1955 (23) that picolinehydroxamic acid methochloride is a particularly active reactivator of inhibited cholinesterase.

Meanwhile, Stewart *et al.* (24), studying the ability to reactivate inhibited cholinesterase of a series of derivatives and relatives of choline, had found that replacement with an alkyl group or a doubly bonded oxygen atom of one or more hydrogen atoms on either of the carbon atoms linking the quaternary nitrogen atom to the hydroxyl group of choline reduced markedly the effectiveness of the compound in reactivating cholinesterase *in vitro*. In view of this finding, it seemed logical, after quaternized hydroxamic acids had been found to be effective reactivators, to convert the ketonic hydroxamate to an aldoxime. This led to 2-formyl 1-methylpyridinium iodide oxime, or 2-PAM, an oxime that has been found to be effective *in vitro* in reactivating inhibited cholinesterase (25–27) and in overcoming neuromuscular block established in the isolated phrenic-diaphragm preparation of the rat by TEPP, DFP or sarin (28). *In vivo*, 2-PAM was found to protect mice against poisoning by paraoxon (29).

A bisquaternary dioxime TMB-4, or 1,3-bis (4-formylpyridinium bromide)-propane dioxime, has been found to reactivate inhibited cholinesterase more rapidly *in vitro* than 2-PAM (30) and to induce more rapid recovery of experimental animals from poisoning by sarin (31). The oximes

MINA (pyruvaldehyde aldoxime) and DAM differ from 2-PAM and TMB-4 in containing no quaternary nitrogen atom. The two former compounds would be expected to cross the blood-brain barrier more readily than the quaternized compounds.

Table 3 compares the effectiveness of 2-PAM, DAM and MINA in reactivating cholinesterase *in vivo* and in antagonizing the lethal effects of a number of cholinesterase inhibitors, both sorts of activity being graded from 3 (greatest effectiveness) to 0 (no definite action). That 2-PAM is more effective in both respects than either MINA or DAM is apparent from the total scores for the three oximes. The failure of any oxime to reduce mortality caused by Malathion or Diazinon may be related to the fact that the oximes were used alone in these experiments. Table 4 shows that a mixture of atropine and 2-PAM is much more effective therapy for poisoning with sarin than is either atropine or oxime alone. If a mixture of atropine and oxime had been used for treating the poisonings by Malathion or Diazinon, the mortality results might have been rather different from those obtained with oxime alone.

Table 4 gives some of our own data for the effectiveness of atropine alone or with 2-PAM and of 2-PAM alone against mortality induced in rabbits by tabun (ethyl N-dimethyl phosphoramido-cyanidate) or sarin. Treatment drugs were injected intravenously within one minute after i.v. administration of the anticholinesterase compound. In both cases, the mixture of 2-PAM and atropine is seen to be the most effective therapy of those tried. Similar findings have been made in the dog (table 5) and in the cat. Table 5 shows also that in the one situation in which two mixtures of atropine and oxime were compared, the mixture of atropine and 2-PAM was much more effective than that of atropine and MINA.

When various oximes were tested (table 6) for ability to promote recovery of the twitch of skeletal muscle in response to stimulation of its motor nerve following intravenous injection of a large dose of sarin, 2-PAM was found to be more active

TABLE 3. EFFECTIVENESS OF OXIMES IN REACTIVATING THE CHOLINESTERASE OF THE BLOOD IN THE RABBIT AND IN REDUCING MORTALITY IN MICE AFTER INJECTION OF ALKYLPHOSPHATE (32)

Alkylphosphate	2-PAM ChE*	2-PAM M†	DAM ChE*	DAM M†	MINA ChE*	MINA M†
Ethyl parathion	3	3	1	0	0	1
Methyl parathion	3	3	0	0	0	0
EPN	2	2	2	1	2	0
TEPP	3	3	0	0	2	0
Pestox-3	2	3	0	1	0	0
Malathion	0	0	1	0	0	0
Diazinon	0	0	0	0	2	0
Dipterex	3	3	1	1	0	1
	16	17	5	3	6	2

* Reactivation of cholinesterases of cells and serum of rabbit blood *in vivo* after reduction by about 35%, graded from 3 (highest) to 0 (lowest) activity.

† Prevention of mortality in mice given about LD65 of alkylphosphate, graded as above.

TABLE 4. EFFECTIVENESS OF 2-PAM AS AN ADJUNCT TO ATROPINE SULFATE IN SAVING RABBITS POISONED BY I.V. INJECTION OF TABUN OR SARIN

Treatment and Dose	LD50-Tabun	LD50-Sarin
None	63 μg/kg	16 μg/kg
Atropine sulfate, 2 mg/kg	160 μg/kg	51 μg/kg
Atropine sulfate, 2 mg/kg and 2-PAM, 5 mg/kg	240 μg/kg	331 μg/kg
2-PAM, 5 mg/kg		24 μg/kg

TABLE 5. ESTIMATED LD_{50}'S FOR DOGS OF ALKYLPHOSPHATES INJECTED SUBCUTANEOUSLY AND FOLLOWED, UPON PROSTRATION OF THE ANIMAL, BY I.V. INJECTION OF ATROPINE SULFATE (2 MG/KG) ALONE OR WITH 2-PAM OR MINA (5 MG/KG)

Alkylphosphate	Atropine	Atropine and 2-PAM	Atropine and MINA
Tabun	650 (6)	1030 (12)	
Sarin	300 (9)	4700 (42)	
EA 1517	30 (12)	500 (9)	
EA 1701	48 (9)	218 (18)	50 (6)

Figures in parentheses are numbers of dogs.

TABLE 6. EFFECTIVENESS OF VARIOUS OXIMES (INJECTED I.V. 5 MIN. AFTER SARIN) IN PROMOTING RECOVERY OF TWITCH RESPONSE OF GASTROCNEMIUS-SOLEUS-TIBIALIS ANTICUS MUSCLE GROUP OF THE CAT TO ELECTRICAL STIMULATION OF SCIATIC NERVE

Oxime	Dose mg/kg	Minutes for 50% recovery	Minutes for 85% recovery
None		19.6	27.1
2-PA	12.5	25.0	
2-PAM	5.0	6.0	8.8
3-PAM	5.0	19.6	30.7
4-PAM	5.0	14.2	19.8
MINA	5.0	13.7	18.8
DAM	35.0	17.5	26.1

2-PA = pyridyl-2-aldoxime; 2,3 or 4-PAM = pyridyl-2,3 or 4-aldoxime methiodide; MINA = pyruvaldehyde aldoxime; DAM = 2,3-butanedione 2-oxime.

than the tertiary oximes MINA, DAM and 2-PA and than its positional isomers 3-PAM and 4-PAM. Furthermore, not only did 2-PAM speed return of the twitch response, as do several other types of compounds, but it also produced much more complete recovery of the tetanic response to repetitive stimuli applied to the sciatic nerve than did other compounds (33).

That the ability of 2-PAM to overcome neuromuscular block induced by cholinesterase inhibitors extends to man has been shown by Grob and Johns (34). Using the adductor muscle of the little finger excited by electrical stimulation of the ulnar nerve, they have found that 2-PAM given after intraarterial injection of sarin leads to rapid return of normal neuromuscular transmission and also inhibits the neuromuscular blocking effect of a second dose of sarin. Grob and Johns found also that the action of 2-PAM is not restricted to P-containing anticholinesterases but applies as well to block of neuromuscular transmission induced by the quaternary amine neostigmine. This finding indicates that 2-PAM may serve the clinician as an antagonist of the profound weakness of the cholinergic crisis encountered during treatment of myasthenic patients with either alkyl phosphate or quaternary amine derivatives. Atropine will be needed still to control the muscarinic phenomena of the cholinergic crisis.

A finding by Grob and Johns that was not expected on the basis of our animal experiments is that DAM also antagonizes the neuromuscular block induced by anticholinesterases in man. DAM has been found to combat in man neuromuscular block induced by neostigmine, bisneostigmine, ambenonium or sarin. The mean i.v. doses of 2-PAM or DAM required for approximately equal effects in these experiments are 675 and 670 mg respectively. Neither oxime had any observable effect on muscarinic symptoms. This latter finding emphasizes again the need for administration of atropine to relieve muscarinic and central neuronal manifestations of anticholinesterase poisoning at the same time that an oxime is given to relieve neuromuscular weakness or paralysis.

Table 7 shows that treatment of animals poisoned by anticholinesterase compounds through administration of atropine alone or accompanied by 2-PAM produced no significantly different effects on cholinesterase in the tissues sampled except in muscle. The asterisk denotes significant difference between the mean reactivations with the two treatments studied. Hobbiger (35) has found that a larger dose of 2-PAM (25 mg/kg i.p.) in the mouse enhances reactivation of cholinesterase in blood, but not in brain, after poisoning by either P-containing or quaternary amine anticholinesterases. Further evidence that oximes have low effectiveness in antagonizing the central neuronal actions of anticholinesterase compounds comes from recent experiments by Wills and Borison in which not only 2-PAM and TMB-4 but also the tertiary oxine DAM, in doses of up to 50 mg/kg, were all found to be impotent to lower the voltage threshold for electrical stimulation of a medullary inspiratory site after it had been elevated by sarin. Atropine was effective in lowering the threshold.

TABLE 7. EFFECTIVENESS OF 2-PAM IN INDUCING REACTIVATION OF CHOLINESTERASE IN BLOOD OF THE DOG AND IN TISSUES OF THE RAT FOLLOWING POISONING BY AN ANTICHOLINESTERASE

	Reactivation by treatment (% amount inhibited)	
	Atropine	Atropine and 2-PAM
Blood	0±8	5±3
Parotid gland	39±12	55±27
Brain	7±1	7±2
Gastrocnemius muscle	24±11	52±17*

Dr. Tatsuji Namba, a Japanese physician, has reported the use of 2-PAM in the treatment of a number of cases of poisoning by parathion or EPN (32, 36). Table 8 presents some of his findings in 25 patients poisoned to varying degrees by parathion. Most of the patients received unspecified doses of atropine in addition to 2-PAM. It is apparent that treatment reduced the number of signs and symptoms observed or experienced in the three groups of patients. It seems possible, also, that the one patient in the 'Slight' group and the three in the 'Moderate' group not given atropine may be responsible for the majority of toxic effects evident in these two groups of patients after treatment. In this regard, it is instructive to compare the toxic effects after treatment in the 'Severe' group, all of whom received both atropine and 2-PAM, with those in the 'Moderate' group, three of whom received no atropine.

Karlog *et al.* (37) have reported the treatment with atropine and 2-PAM of a case of severe parathion poisoning of suicidal origin (estimated retained dose of parathion by mouth: 1.75 gm). The patient required artificial ventilation intermittently during the first 5 hours after poisoning; during this time he received i.v. 96 mg of atropine

TABLE 8. EFFECTIVENESS OF TREATMENT WITH 2-PAM, WITH OR WITHOUT ATROPINE, IN REMOVING THE SYMPTOMS OF PARATHION POISONING IN MAN (36)

	Slight		Moderate		Severe	
	Pre	Post	Pre	Post	Pre	Post
Nausea	2	1	14	3	7	
Pallor	3		7	4	8	1
Vomiting	1		10		8	
Salivation	2		7		6	
Headache	2	1	10	6	3	
Vertigo			9	2	5	
Paresthesia	1		7	2	6	
Fasciculation			6		7	
Sluggish light reflex			5	1	5	1
Impaired speech			6	2	4	
Cramp			5		4	
Miosis			5	3	4	
Dyspnea			1		4	

Slight: 3 patients given mean dose of 1.2 gm of 2-PAM, i.v., at mean of 2.8 hr after parathion; 2 given atropine.

Moderate: 14 patients given mean dose of 1.1 gm of 2-PAM at mean of 3.3 hr after parathion; 11 given atropine.

Severe: 8 patients given mean dose of 1.1 gm of 2-PAM at mean of 3.9 hr after parathion; 8 given atropine.

sulfate. Spontaneous respiration at the rate of 40/min. was resumed at about 5 hours after poisoning and never failed thereafter.

The patient received i.v. an additional 178 mg of atropine sulfate between 5 and 11.5 hours after the poisoning and another 80 mg s.c. during the succeeding 13.5 hours. Between 5.7 and 6.9 hours after poisoning, between 9.9 and 11.3 hours and between 59.3 and 63.9 hours the patient received three infusions of 3.5 gm of 2-PAM in saline. Each infusion brought about sharp increases in both plasma and red cell cholinesterase activities, but these reactivations of cholinesterase persisted for only a few hours after the end of each infusion. The patient's plasma cholinesterase began to rise steadily, but slowly, on the second hospital day; but a progressive increase in red cell cholinesterase activity was not definite until about the tenth hospital day.

On the fourth hospital day, and for two days thereafter, the man was given 300,000 units of penicillin per day for pneumonia. The period of treatment with penicillin corresponds to a prolonged elevation of red cell cholinesterase activity ending between the seventh and eighth hospital days. Whether this elevation of red cell cholinesterase was due to the penicillin or to some other factor is unknown at present; there is here the suggestion, at least, that the action of penicillin on inhibited cholinesterase may be worthy of study.

TABLE 9. TOXIC EFFECTS OF OXIMES IN MAN (38)*

	DAM†	2-PAM‡	TMB-4§
Dizziness	37	100	
Blurred vision	21	71	
Nausea	11	18	
Diplopia		47	
Headache		24	
Unconsciousness	32		
Convulsions	11		
Hypotension			100
Tachycardia			50
Urinary excretion (4 hr.)	3(2)	83(3)	22(2)

* Figures are per cent of subjects displaying each effect, except for last item. There the figures are mean urinary excretions of the oxime, in per cent of the amount of oxime injected i.v., and figures in parentheses are the numbers of subjects used in the urinary excretion studies.

† Dosage 15 to 30 mg/kg, i.v.; 19 subjects.

‡ Dosage 15 to 20 mg/kg, i.v.; 17 subjects.

§ Dosage 15 to 20 mg/kg, i.v.; 2 subjects.

Table 9 shows toxic effects experienced or observed in patients given various oximes. TMB-4 has been given to only two patients because of the profound hypotension and temporary anuria induced by it. DAM and 2-PAM have been given to more adequate numbers of subjects. It is apparent that DAM is more likely to exert serious effects on the central nervous system than is 2-PAM, and probably than is TMB-4. It is apparent also that 2-PAM is excreted in the urine more rapidly than TMB-4 and, especially, DAM. The latter finding probably is an important factor contributing to the superiority of DAM and TMB-4 over 2-PAM as a prophylactic agent in anticholinesterase poisoning (17, 31).

An interesting recent finding is that combinations of oximes may be more effective than either oxime alone. Edery and Schatzberg-Porath have reported (39) that a mixture of DAM and 2-PAM has greater therapeutic activity than either compound alone against TEPP or Dimefox, but not against paraoxon or DFP. Wilson has reported (40) somewhat similar activity by a mixture of PAD (2-formyl 1-dodecylpyridinium iodide oxime) and 2-PAM used as an adjunct to atropine in therapy of poisoning by sarin.

SUMMARY

Oximes, such as DAM, 2-PAM and TMB-4, are effective adjuncts to atropine in the treatment of poisoning by anticholinesterase com-

pounds, whether of the alkylphosphate or the quaternary amine type. These oximes seem to be particularly active in restoring neuromuscular transmission, presumably by reactivation of inhibited cholinesterase at the neuromuscular junction. The oximes, therefore, afford to the clinician a means for controlling the nicotinic phenomena of the cholinergic crisis in myasthenic patients under treatment with anticholinesterase drugs and in patients poisoned accidentally or purposely with the same type of compound. The tertiary oxime DAM and the quaternary oxime TMB-4, both of which are excreted from the body more slowly than the quaternary oxime 2-PAM, have significant prophylactic activity in experimental anticholinesterase poisoning. Mixtures of oximes (as, DAM and 2-PAM) may be more effective adjuncts to atropine than either oxime alone.

REFERENCES

1. Hottinger, A. and H. Bloch. *Helv. Chim. Acta* 26: 142, 1943.
2. Berry, W. K. and A. Todrick. *Porton Tech. Pap.* 91, Dec. 1948.
3. Koster, R. *J. Pharmacol. & Exper. Therap.* 88: 39, 1946.
4. Koelle, G. B. *J. Pharmacol. & Exper. Therap.* 88: 232, 1946.
5. McNamara, B. P., G. B. Koelle and A. Gilman. *J. Pharmacol. & Exper. Therap.* 88: 27, 1946.
6. Augustinsson, K.-B. and D. Nachmansohn. *J. Biol. Chem.* 179: 543, 1949.
7. Cohen, J. A., M. G. P. J. Warringa and B. R. Bovens. *Biochim. et biophys. acta* 6: 469, 1951.
8. Augustinsson, K.-B. and M. Grahn. *Acta physiol. scandinav.* 27: 10, 1952.
9. Augustinsson, K.-B. *Arkiv för kemi* 6: 331, 1953.
10. Fleisher, J. H., J. P. Corrigan and J. W. Howard. *Brit. J. Pharmacol.* 13: 291, 1958.
11. Jaco, N. T. and D. R. Wood. *J. Pharmacol. & Exper. Therap.* 82: 63, 1944.
12. Jandorf, B. J., *Med. Labs. Res. Rep.* No. 81, 1951.
13. Augustinsson, K.-B., *Acta chem. scand.* 6: 959, 1952.
14. Hackley, B. E., Jr., R. Plapinger, M. Stolberg and T. Wagner-Jauregg. *J. Am. Chem. Soc.* 77: 3651, 1955.
15. Hackley, B. E., Jr., Thesis, University of Delaware, Newark, Delaware, 1956.
16. Green, A. L. and B. Saville. *J. Chem. Soc.* 1956: 3887.
17. Koon, W. S., N. B. Billups and F. W. Oberst. Chemical Warfare Labs. Report No. 2146, 1957.
18. Askew, B. M. *Brit. J. Pharmacol.* 11: 417, 1956.
19. Askew, B. M., *Brit. J. Pharmacol.* 12: 340, 1957.
20. Wilson, I. B. *J. Biol. Chem.* 190: 111, 1951.
21. Hobbiger, F. *Brit. J. Pharmacol.* 6: 21, 1951.
22. Wilson, I. B. and E. K. Meislich. *J. Am. Chem. Soc.* 75: 4628, 1953.
23. Wilson, I. B. *Discussions Faraday Soc.*, 20: 119, 1955.
24. Stewart, W. C., J. Scoffield and K. Siluch. Defence Research Board Extramural Grant 354, Dec. 1953.
25. Davies, D. R. and A. L. Green. *Discussions Faraday Soc.*, 20: 269, 1955.
26. Wilson, I. B. and S. Ginsburg. *Biochim. et biophys. acta* 18: 168, 1955.
27. Childs, A. F., D. R. Davies, A. L. Green and J. P. Rutland. *Brit. J. Pharmacol.* 10: 462, 1955.
28. Holmes, R. and E. L. Robins. *Brit. J. Pharmacol.* 10: 490, 1955.
29. Kewitz, H. and I. B. Wilson. *Arch. Biochem.* 60: 261, 1956.
30. Poziomek, E. J., B. E. Hackley, Jr. and G. M. Steinberg. *J. Org. Chem.* 23: 714, 1958.
31. Bay, E., S. Krop and L. F. Yates. *Proc. Soc. Exper. Biol. & Med.* 98: 107, 1958.
32. Namba, T., XIII Congress of the Japanese Association of Public Health, Oct. 1958.
33. Wills, J. H., A. M. Kunkel, R. V. Brown and G. E. Groblewski. *Science* 125: 743, 1957.
34. Grob, D. and R. J. Johns. *Am. J. Med.* 24: 497, 1958.
35. Hobbiger, F. *Brit. J. Pharmacol.* 12: 438, 1957.
36. Hiraki, K., T. Namba et al. *Naika no Ryôiki* 6: 84, 1958.
37. Karlog, O., M. Nimb and E. Poulsen. *Ugesk. laeger* 120: 177, 1958.
38. Jager, B. V., DA 18-108-cml-5421, April 1958.
39. Edery, H. and G. Schatzberg-Porath. *Science* 128: 1137, 1958.
40. Wilson, I. B. *Biochim. et biophys. acta* 27: 196, 1958.

CELL MEMBRANE AS SITE OF ACTION OF HEAVY METALS[1]

ASER ROTHSTEIN

Departments of Radiation Biology and Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, New York

TOXIC CHEMICAL substances act ultimately by chemical reactions with biochemical substances in cells. In consequence, a cellular function is disturbed which manifests itself in an observed toxicological response in the animal. The chain of events between the original chemical insult and the final observed response, may be so very complicated that one might seriously doubt that toxicological actions can ever be traced back to initial chemical events. Fortunately, they sometimes can. All too often, however, the limiting factor is the fund of knowledge concerning those normal patterns of cellular function at the chemical level, which can be disturbed by the toxic agents. For this reason, toxicological studies at the cellular level are doubly useful, leading not only to information concerning the mechanism of the toxic response, but also to fundamental biological information concerning cellular function. Biochemists and physiologists have long recognized this point in their use of toxic agents as inhibitors of specific functions, using agents whose chemical interactions with biologically important molecules are fairly well understood. Toxic agents in this sense are research tools for exploring the cell and its chemistry. That heavy metals can often serve this function, is the premise of the present discussion.

In a strictly chemical sense, interactions of heavy metals with biologically important molecules are fairly well understood (1). In the case of proteins, interactions are of two general types, those associated with the presence of specific ligands, particularly carboxyl, imidazole and sulfhydryl groups, and those associated with special arrangements of amino acid residues. The latter are of particular importance in enzyme reactions. In the case of nucleic acids, the phosphoryl groups are of primary importance because of their ability to form chelates with metallic cations (2).

Because of their interactions with ligands present in all proteins, heavy metals are particularly potent enzyme inhibitors. Indeed, a listing of the enzyme systems known to be sensitive to heavy metals would include all of the essential metabolic machinery of the cell. This does not imply that all enzymes are equally sensitive. The sensitivity and mechanism of inhibition vary considerably with particular enzymes and with particular heavy metals. But the important point is the general lack of specificity. In fact, the inhibitory propensities of most heavy metals is so widespread that predictions of cellular effects from known actions on purified enzymes are most difficult. There are too many potential enzyme-targets in the cell.

In order to evaluate toxicological responses of cells, it is necessary to consider not only the chemical reactions of biologically important molecules, but also the structure, and the chemical composition of cells. Studies carried out in test tubes are well defined in a chemical sense. Solutions of known compositions are used, usually containing a highly purified protein, perhaps a buffer, a substrate and the heavy metal. The cell on the other hand, is an exceedingly complex structure containing an endless variety of substances including, of course, its many enzymes At first glance the situation seems hopelessly complicated from a chemical point of view. Nevertheless, certain predictions can be made concerning the actions of the heavy metals. Consider the sequence of events following the addition of a heavy metal salt to a suspension of cells. The chemical composition of the medium can be experimentally controlled within certain limits so the chemical state of the metal ion in the extracellular solution is known. The metal ions in the medium will tend to diffuse into the interior of the cell, interacting as they pass inward with any reactive cytoplasmic sites. The first generalization, then, is the obvious but important one that the metal acts from the outside toward the inside. The first sites of interaction will be at the surface boundaries of the cell, followed, as the metal diffuses into the cell, by reactions within the interior of the cell. The factors that must be taken into account are both

[1] This paper is based in part on work performed under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project. Support was also received from the Smith, Kline & French Foundation and from the Electrometallurgical Company, a division of Union Carbide and Carbon Corporation.

physical, in terms of diffusion barriers, and chemical, in terms of the metal-affinities of various chemical groups, their concentrations and the rates of the reactions.

In the system outlined above, the cell membrane plays a primary role. First, it is exposed directly to the extracellular concentration of metal. Any chemical ligands of the membrane capable of interacting will do so. If the cell, thereby, suffers irreparable physiological damage, subsequent actions on internal cellular systems are of secondary importance. Secondly, the membrane may offer a major physical resistance to the diffusion of the metal into the interior of the cell. On this basis, a second generalization can be made that the cytoplasmic systems are protected to some extent by the cell membrane. They are subjected only to the amounts of metal which can pass through the cell membrane during the time of exposure. Other diffusion barriers within the cell may also play a role.

Within the cytoplasm, many substances present are capable of combining with the metals. Many of these are 'physiologically inert' insofar as any particular function is concerned. Thus a third generalization can be made, that the 'physiologically inert' sites, by soaking up metal ions, will to some extent protect the 'physiologically active' sites from damage. Because of the combined protective effects of the membrane and of 'inert' cytoplasmic substances, metabolic enzymes are usually far less sensitive *in vivo* than *in vitro*. The most probable site of damage of metals is therefore the membrane itself, rather than the enzymes inside the cell.

From an experimental point of view, two aspects of the action of heavy metals on cell membranes are of importance. The first is the nature of the membrane-metal reaction in a chemical sense. This is determined by the nature of the chemical entities present in the membrane, with which the metal can react. The second is the nature of the physiological disturbance, if any, resulting from the chemical alterations in the membrane. The most useful experiments are those in which correlations can be made between the chemical reaction and the physiological effect. Inasmuch as knowledge of both the molecular structure and function of the membrane is somewhat limited, it can be stated again that studies with heavy metals are doubly useful. They provide insight into the nature of heavy metal toxicity, and they also provide additional information concerning membrane structure and function.

The recent literature contains many examples of the use of heavy metals in the evaluation of membrane function. In the present discussion no attempt will be made to list or to discuss the many excellent studies. Rather, a very few experiments have been selected which illustrate certain principles, and which illustrate some of the experimental techniques that can be applied.

ACTION OF MERCURY AND COPPER ON MUSCLE CELLS

When the excised diaphragm of the rat is exposed to mercury or copper (as the chlorides) two kinds of measurements can be made: *a*) the binding of the metal to the muscle and *b*) the interference with physiological functions (3). The binding of mercury as shown by the disappearance curves (fig. 1) can be divided into two phases: one rapid and one slow. The rapid phase has a half time of a few minutes and is essentially complete in 20 minutes. The slow phase shows up

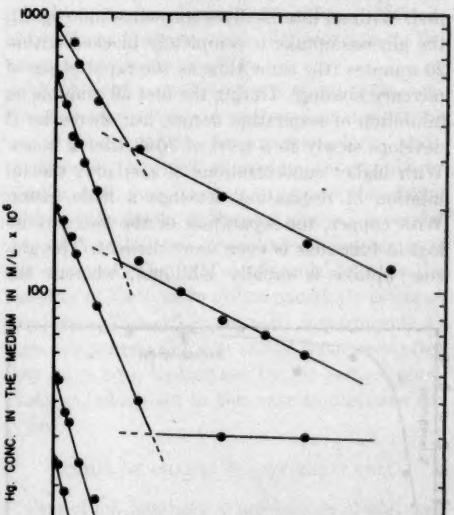


FIG. 1. Semi-logarithmic plot of uptake of isotopic mercuric chloride by rat diaphragm. Ten ml of each concentration of $HgCl_2$ in Krebs' Ringer phosphate buffer, pH 7.4. Weight of diaphragms 600 mg, temperature, 38°C, in oxygen.

only at higher concentrations of mercury and has a half time of about 60 minutes.

If the muscle is removed from the mercury and placed in a solution containing cysteine (a complexing agent for mercury), some of the bound metal can be removed. If the exposure to mercury was short (less than 20 minutes) and the concentration relatively low, almost all can be removed in 30 minutes, but if the exposure is longer and the concentration higher, only a small fraction comes out in 30 minutes. It is apparent, then, that the mercury represented by the rapid phase of uptake is easily mobilized whereas that represented by the slow phase is difficult to mobilize.

Two physiological responses to the muscle were measured—the ability to absorb glucose and the ability to respire. (In muscle, these responses are independent of each other. Respiration involves only endogenous substrates. Absorbed glucose passes only into the glycogen pool but not directly to respiratory pathways.) With low concentrations of mercury, in which only the rapid binding occurs, only the glucose uptake is inhibited. With an intermediate concentration (fig. 2), the glucose uptake is completely blocked within 20 minutes (the same time as the rapid phase of mercury binding). During the first 20 minutes no inhibition of respiration occurs, but thereafter it develops slowly to a level of 30% after 2 hours. With higher concentrations of mercury, the inhibition of respiration develops a little faster. With copper, the separation of the two physiological functions is even more distinct. The glucose uptake is rapidly inhibited, whereas the respiration is diminished only after a lag period of 80 minutes. The respiration eventually (after several hours) is inhibited to a greater extent than is the glucose uptake.

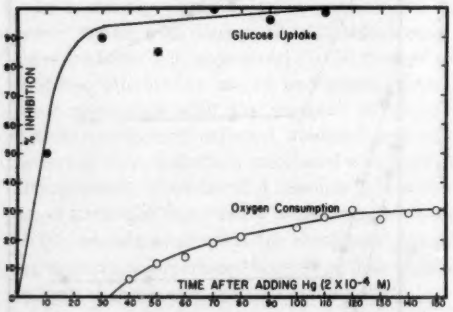


FIG. 2. Comparison of inhibition, by mercuric chloride, of aerobic glucose uptake and of respiration of rat diaphragm. Time is expressed in min. after adding $HgCl_2$. Average weight of diaphragm about 300 mg. Total volume 3 ml, Krebs' Ringer phosphate buffer, pH 7.4, glucose concentration 100 mg %, temperature, 38°C, in oxygen.

The two physiological effects, inhibition of glucose uptake and of respiration, can be identified with two phases of mercury binding, on the basis of the mercury concentrations required, and on the basis of the time sequence. A further correlation is the action of cysteine which can remove the mercury associated with the rapid phase, and also reverses the inhibition of sugar uptake, but which does not remove the mercury associated with the slow phase and cannot reverse the inhibition of respiration. In connection with the latter observation, it is interesting to note that the respiration of homogenates of muscle is rapidly inhibited by mercury and rapidly reversed by cysteine. The slow, irreversible inhibition of respiration in the intact muscle cell is due to cellular structure rather than to any inherent properties of the enzyme systems.

What are the geographical sites of the two actions of mercury on the cell? Studies on sugar uptake in many kinds of cell, including muscle, suggest that sugar combines with a receptor in the membrane which, by an unknown mechanism, allows entry into the cell. Respiration on the other hand, is associated with the mitochondria which in muscle lie between the contractible fibrils and the membrane. These two locations are compatible with all of the data presented above. The action on the membrane (sugar uptake) is rapid and can be reversed by extracellular substances (cysteine). The action on the interior of the cell (respiration) is slow to develop, requires higher concentrations, and cannot be reversed by extracellular agents.

The studies of mercury and muscle provide a particularly good illustration of the concept of the outside to inside action of a toxic agent, and also of the protective action of the cell membrane as a diffusion barrier. The experiment with homogenates also illustrates the potential protective action of the cytoplasmic constituents. The concentration of mercury necessary to inhibit the respiration of the homogenate is almost 200 times as high as that required to achieve the same inhibition in the intact cell. In the intact cell, the mercury as it diffuses past the membrane, next comes in contact with the mitochondria which lie close under the membrane, inhibiting the respiration. The contractile system, being more centrally

located does not have the same opportunity to combine with the mercury. In the homogenate, on the other hand, the actomyosin is uniformly distributed. It soaks up large amounts of the added mercury. Therefore, larger concentrations are required for the inhibition of respiratory enzymes.

INHIBITION OF ENZYMES IN THE CELL SURFACE

A number of cellular enzymes are located on the surface of the cell (4). Because of their location, such enzymes are directly accessible to metal ions in the extracellular fluid, and may therefore be inhibited in almost the same way as the purified isolated enzyme. For example, phosphatases of the intact yeast cell membrane can be inhibited by molybdate or tungstate (fig. 3) in concentrations as low as 10^{-7} M (5). If some cells are broken and the contents liberated, much higher concentrations are required because of the protective action of cellular substances. The kinetics of inhibition are typical of those found with purified enzymes. For example, in figure 4, the reciprocal of the rate of enzyme activity is plotted against the reciprocal of the substrate concentration. The data can be fitted by straight lines indicating that the Michaelis-Menton equation (derived from mass-law relationship) is applicable. The intercept on the 1/R axis represents the maximal rate of the enzyme reaction when it is 'saturated' by high substrate concentrations. Because the lines for data in the presence and absence of molybdate passes through the same intercept, the inhibition can be classified as 'competitive'. That is, the inhibitor competes with the substrate for the reactive site on the enzyme.

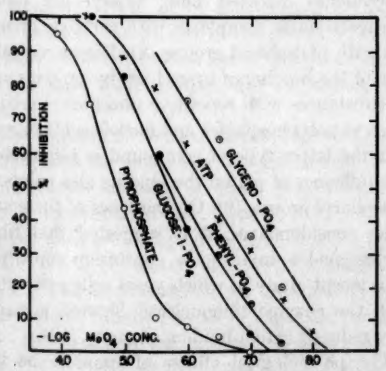


FIG. 3. Inhibition by molybdate of hydrolysis of various phosphate compounds by yeast cells. Inhibition was calculated from inorganic phosphate liberated in 10 min. by a yeast suspension containing 10 mg wet weight per ml of suspension. Substrate concentrations were 10^{-3} M; the pH was 3.5.

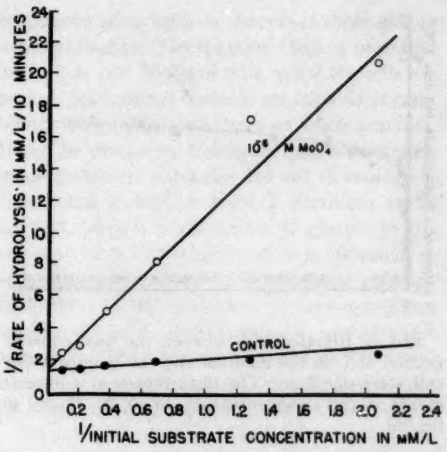


FIG. 4. Kinetics of molybdate inhibition of pyrophosphate hydrolysis by cell-surface phosphatase. Yeast concentration was 10 mg/ml and the pH 3.0.

The properties and functions of cell surface enzymes can be directly determined, *in vivo*, by the use of heavy metals. For example, if the phosphatases in the yeast membrane are completely blocked with molybdate or tungstate, the ability of the cell to divide, to ferment glucose, to respire glucose, or to absorb phosphate or potassium is not impaired. The only measurable defect is the inability of the cells to utilize phosphate esters as substrates. The cell is normally impermeable to such compounds and can utilize them only after they have been hydrolyzed by the surface phosphatases, which act in this case as digestive enzymes.

ACTION OF URANYL ION ON YEAST CELL

Uranyl ion has been a particularly useful tool because it acts primarily on the membrane. If yeast cells are exposed to solutions of uranyl nitrate, at an acid pH (to avoid formation of hydroxide), a fraction of the uranium is immediately taken up (2 minutes is the most rapid determination). After the initial rapid phase, very little additional metal is absorbed (6). It is the rapid uptake and its effects with which the following experiments are concerned.

The amount of uranium that is taken up in

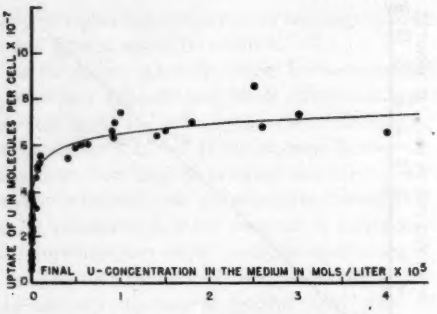


FIG. 5. Relationship between the final concentration of U in the medium and the U-uptake per cell after one hour. The data represent 4 experiments at yeast concentrations of 10, 20, 30 and 40 mg/ml.

relation to the uranium concentration follows the curve of figure 5. At low concentrations the metal is almost completely absorbed, but as the concentration is increased a maximal uptake of approximately 1×10^{-3} M/kg of cells is attained which represents the saturation of certain binding sites. The uranium studies provide useful information concerning the chemical nature, the cellular location and the physiological function of these sites.

The binding curve (fig. 5) can be fitted by a simple mass law relationship:

$$U + Y \rightleftharpoons UY \qquad (1)$$

$$K = \frac{(U)(Y)}{(UY)} \cdot \frac{fu.fy}{fuy} \qquad (2)$$

where (U) is the concentration of free uranyl ion, (Y) is the concentration of free binding sites, (UY) is the bound uranium, and fu, fy and fuy are the activity coefficients. In the dilutions used, fu is essentially 1.0. The activity coefficients for the solid phases fy and fuy are unknown, but it is assumed that the ratio fy/fuy is constant and the term is lumped in the constant K_1. The data (table 1) agree with the equation very closely, giving a K_1 which is remarkably constant at about 4×10^{-7} (7).

According to *equation 1*, the binding of uranyl ion is completely reversible. That such is the case is demonstrated by competition studies. All of the other cations tested can displace uranium from the binding sites by direct competition. However, uranyl ion forms by far the most stable complex (300 times as stable as Ca^{++} and Mg^{++}, and 10,000 times as stable as Na^{+} and K^{+}).

TABLE 1. MASS LAW CONSTANTS FOR BINDING OF URANIUM BY YEAST

$U_t \times 10^{-5}$ M	(U) $\times 10^{-7}$ M	(UY) $\times 10^{-6}$ M	(Y) $\times 10^{-5}$ M	$K_1 \times 10^{-7}$ M
0.8	0.18	0.78	1.92	4.4
1.6	0.26	1.57	1.84	3.0
2.4	0.49	2.35	1.77	3.7
3.2	0.75	3.13	1.69	4.1
4.0	0.86	3.91	1.61	3.5
4.8	1.08	4.69	1.53	3.5
6.0	1.47	5.85	1.41	3.5
6.8	1.71	6.63	1.34	3.4
8.0	2.89	7.71	1.23	4.6
16.0	14.40	14.60	0.54	5.3

* Yeast concentration was 20 mg/ml in each case, equivalent to a concentration of binding sites (Y_t) of 2×10^{-5} M.

† Values are based on averages of duplicate determinations.

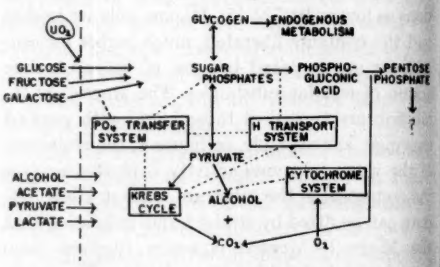


FIG. 6. Scheme of metabolism.

What is the chemical nature of the binding sites? Obviously they are negatively charged groups. In mildly acid solutions as used in the experiments reported here, uranyl ion forms relatively stable complexes with carboxyl groups and with phosphoryl groups (8). Direct comparisons of the binding of uranyl ion by proteins and by substances with repetitive phosphoryl groups (such as polyphosphates and nucleic acid) suggest that the latter type of compound is responsible. The influence of pH on the binding also points to phosphoryl groups (9). On the basis of these and other considerations, it is suggested that ribonucleic acid is involved, a conclusion supported by a recent study in which yeast cells pretreated with the enzyme ribonuclease, showed a markedly reduced cation-binding capacity (10).

The physiological effects of uranium on the yeast cell can be described best by a diagram (fig. 6). The yeast can normally respire or ferment three hexoses, glucose, mannose, and fructose. In the presence of uranium none of the sugars can be used either for respiration or for fermentation. Yet the respiratory system itself is unaffected,

TABLE 2. INHIBITION OF FERMENTATION BY URANIUM AND REVERSAL BY EXTRACELLULAR PHOSPHATE

UO_2^{++} Conc. μmole/l	Extracellular Inorganic Phosphate μmole/l	Inhibition %	Intracellular Inorganic Phosphate μmole/l
00	00	00	10,600
20	00	91	8,300
20	50	83	9,200
20	100	69	9,100
20	200	30	9,700

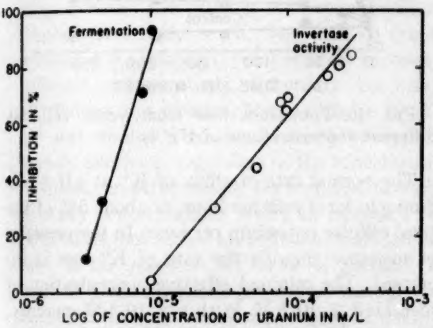


FIG. 7. A comparison of the inhibitory effects of uranium on fermentation of glucose and on invertase activity of living cells. Yeast concentration was 20 mg/ml. The glucose concentration was 0.1 M and the sucrose concentration, 0.16 M.

for the uranium-treated cells can respire alcohol at normal rates. The glycolytic system is also unimpaired, for the uranium treated cells are capable of fermenting internal stores of glycogen (induced by DNP) or of synthesizing glycogen from alcohol. Nor are any of the phosphorylating systems altered. Uranium has a specific effect on the reactions directly involving the sugars (11, 12).

The quantitative relationship between uranium binding and the inhibition of fermentation is of some interest, because a direct proportionality can be demonstrated (13). That is, if 50% of the binding sites are occupied by uranium, the inhibition is 50%. If 75% are occupied, the inhibition is 75%. This implies that a site occupied by uranium cannot function at all in sugar uptake. On the other hand, if the uranium is displaced by Mg^{++}, Ca^{++}, K^+ or Na^+, the sugar uptake is normal. For this reason, the reaction inhibited by uranium cannot be the phosphorylation of sugar by hexokinase, the first step in glucolysis. This enzyme requires Mg^{++} and is inhibited by Ca^{++} (14).

The location of the uranyl binding sites in the cell throws some light on the role of these sites in sugar utilization. The maximal binding of uranyl ion is 1×10^{-3} M/kg of cells, yet if the cells are broken, the internal contents are capable of combining with almost 100 times as much uranium. It can be concluded, therefore, that the uranium binding sites are not inside the cell. A conclusive experiment is that of table 2. Uranium at 20 μmole/l. inhibits fermentation of glucose to the extent of 91%. The addition of 50 to 200 μmole/l. of inorganic phosphate to the medium, substantially reverses the inhibition. Yet the cytoplasm already contains 10,000 μmole/l. of inorganic phosphate. The uranium is bound on the membrane where it is in contact with the extracellular, and not the intracellular environment (5).

In view of the location of the uranium sites on the membrane, the nature of the physiological response and the knowledge of the mechanisms by which sugars enter cells, it has been suggested that uranium acts by blocking sugar receptors which are responsible for the transfer of sugars through the membrane to the enzymatic sites of its breakdown (15).

Uranium may act on the membrane in another way if the concentration is sufficiently high. In figure 7, the inhibition of fermentation of glucose is complete if the concentration of the metal is 1×10^{-5} M. Above this concentration a second, distinct physiological effect is observed, the inhibition of the enzyme invertase (which is located on the cell membrane). The sharp separation of the two inhibitory effects indicates that two kinds of sites are involved, with different affinities for uranium (16). It has already been pointed out that the fermentation sites are phosphoryl groups. Studies on the action of uranyl ion on pure invertase indicate that the sites of inhibition in this case are free carboxyl groups of the enzyme (17).

The binding studies with uranium also suggest

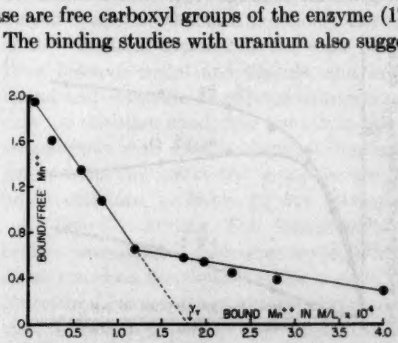


FIG. 8. Mass-law plot of Mn^{++} binding by the yeast cell.

the existence of two sites. With high concentrations of the metal, the amount of metal that is bound is greater than can be accounted for by the phosphoryl groups. If Mn^{++} binding is studied rather than UO_2^{++}, the existence of two kinds of sites is even more readily apparent (7). Figure 8 represents a mass-law plot of the Mn-data, based on *equation 1*. The steep slope represents the binding by phosphoryl groups and the intercept the number of phosphoryl sites (1×10^{-3} M/kg, the same as determined by UO_2^{++}-binding). The shallow slope represents the carboxyl-binding, which has a higher dissociation constant (the slope is equal to 1/k). The number of carboxyl groups can be estimated only very roughly because of the extrapolations involved at approximately 3×10^{-3} M/kg of cells.

BREAKDOWN OF YEAST MEMBRANE TREATED WITH MERCURY

The potassium content of yeast cells (and of other cells) is high, of the order of 0.15 M/kg of cells. Yet, the cells suspended in distilled water lose K^+ only very slowly, due to the impermeability of the membrane. The potassium level of the cell is determined by two factors, the rate of loss (or efflux), dependent on the permeability of the membrane to K^+, and the rate of uptake (or influx) dependent on a metabolism dependent transport (18). Ordinarily the determination of the separate contributions of the two factors is difficult without resorting to isotope studies. However, the efflux of K^+ can be conveniently studied by the cell column technique in which K^+-free solutions are passed rapidly through suspension. This technique prevents the accumulation of K^+ in the medium, and the initiation of active absorption mechanisms (19).

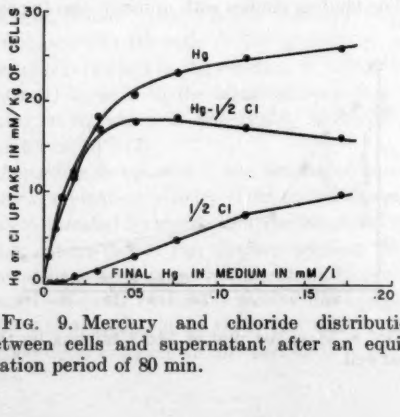


FIG. 9. Mercury and chloride distribution between cells and supernatant after an equilibration period of 80 min.

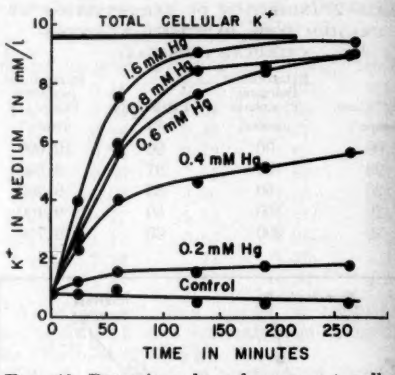


FIG. 10. Potassium loss from yeast cells at different concentrations of $HgCl_2$.

The normal rate of efflux of K^+ at pH 4.5 is 8 mmole/kg of cells per hour, or about 5% of the total cellular potassium per hour. In the presence of mercuric chloride the rate of K^+ loss is increased. The minimal effective concentration of metal is 3×10^{-6} M. With 1×10^{-4} M mercury, the rate of loss is almost 100 mmole/kg/hr., almost 15 times that of the control, or 70% of the cellular K^+ in one hour. It is this increased permeability to potassium that will be discussed. As in the other studies, both the binding of the heavy metal to the cell, and the physiological response (K^+ loss) were determined.

The binding of mercury by yeast cells is different in several respects from the binding of cations described in the previous section. An equilibrium distribution of the mercury is observed, but the rate of binding and of desorption is relatively slow (20). For example, maximal binding occurs after 15–20 minutes (compared with less than 2 minutes for UO_2^{++} and other cations). Furthermore, desorption is also slow, and very dependent on temperature. At 38° C, desorption (in the presence of an excess of cysteine) is 90% complete, whereas at 7.2° C, it is only 40% complete in 3 hours. In contrast, UO_2^{++} binding can be completely reversed in less than 2 minutes in the presence of a surplus of a complexing agent such as phosphate.

Not only are the kinetics of binding and desorption of mercury different from those of the other cations, but the amounts of mercury that can be bound are considerably larger. The binding curve (fig. 9) shows two phases, one in which the metal alone is bound and a second in which chloride is taken up together with the metal. The steep phase involves 18 mmole of Hg per kg of

cells, in contrast to 1 mmole for the binding of UO_2^{++} or other cations by phosphoryl groups (fig. 5). Obviously, then, the ligands which bind mercury are not the same as those which bind UO_2^{++}. A variety of evidence, which is reviewed elsewhere (20, 21) suggests that the binding of mercury associated with the physiological defect (K^+-loss), is due to sulfhydryl groups in the cell membrane.

No simple stoichiometric or mass-law relationship between the binding of mercury and the physiological response (K^+ loss) can be demonstrated. For example, if yeast cells are exposed to different concentrations of mercury, the loss of K^+ follows the pattern shown in figure 10. The K^+ loss reaches a maximal value after approximately one hour, regardless of the concentration of metal. In other words, the response to higher mercury concentrations is not an increased rate of K^+-loss, but rather an increased maximal K^+-loss. If the response (in terms of maximal K^+ loss) is plotted against the log of the mercury concentration, a symmetrical S-shaped curve is found that corresponds to the integrated form of the 'normal distribution'.

One possible explanation for the observed response to mercury is that individual cells respond in an 'all or none' fashion. At any given mercury concentration, a cell is either not affected at all, or it becomes completely leaky. That such is the case is suggested by the following observations: *a*) If cells exposed to mercury are treated with appropriate concentrations of a dye such as Methylene Blue, a fraction of the cells becomes stained, whereas unexposed cells do not stain. An almost perfect correspondence is found between the fraction of the cells that are stained and the fractional loss of K^+ from the population of cells. *b*) A similar relationship can be demonstrated for K^+-loss and the ability of the cells to form colonies. It is apparent, then, that these cells which become stained and those cells which cannot divide, are the same cells which have lost all their potassium. Each cell has a threshold which, if exceeded, results in the inability to retain K^+. The S-shaped 'normal' curve relating log of mercury concentration and maximal K^+-loss, represents the distribution of thresholds within the population.

The defect in the membrane produced by mercury is not specific for potassium, but represents a general breakdown in the permeability of the membrane. Not only does K^+ escape, but phosphate compounds, amino acids and nucleotides as well. Other agents, such as the basic redox dyes, as well as radiation with x-ray or U.V. light, produce the same effect (22). In fact, some of the observed actions of these agents on metabolism may be secondary effects, which result from the loss of cellular constituents through the damaged membrane.

The membrane defect produced by mercury results from an interaction of the metal with membrane ligands. Below a mercury concentration of 0.1 mM, virtually no K^+-loss is observed. Yet an appreciable amount of mercury is bound by the cells (2 mmole/kg). At a mercury concentration of 1.6 mM, the K^+-loss is virtually 100%. The amount of mercury bound by cells is 12 mmole/kg. But no particular inflection is seen in the binding curve (fig. 9). In fact the steep phase continues to a binding of 18 mmole/kg. It is obvious then that the binding curve of figure 9 represents chemically similar ligands, some of which are physiologically responsive (in terms of membrane permeability). This diversion of the metal by inert sites adds to the difficulties of interpretation of the mechanism of action of the heavy metal.

The nature of the response curve itself is of some interest. In the previous sections, the actions of molybdate on phosphatases, and of UO_2^{++} on fermentation and on invertase activity could be analyzed in terms of mass law derivations similar to those used for describing inhibition of enzymes. (Many other responses to heavy metal fit in this category.) Why is the response to mercury so different, giving a population curve of the type found with 'all or none' reactions? The answer lies in the nature of the physiological responses. In the case of the molybdate and UO_2^{++}, the metal interacts reversibly with receptors involved in specific chemical functions. The extent of the inhibition depends on the mass law reactions between metal and ligands, and between ligand and substrate or effector substance. Mercury, on the other hand, does not act, in this case, on receptors with specific chemical functions. It reacts with any sulfhydryl group in the membrane structure, probably by the formation of —S—Hg—S— bridges. The formation of such bridges constitutes a molecular stress. When the stress reaches a threshold level for a given cell, a generalized breakdown of the membrane results, with the release of cellular constituents. Although the mercury can still be desorbed from the cell, the physiological insult cannot be reversed. For this reason, no stoichiometric relation of response

and metal-binding is apparent, nor does the response follow any mass law formulation.

OTHER STUDIES

Only a few illustrative studies have been described. Many others have provided useful information concerning both the toxic action of the metals and the functions of the cell membrane. Some of these can be listed: the inhibition of glycerol transfer in the red blood cell by copper (23) and mercury (24); the changes in K^+ permeability and transport in the red cell induced by Pb, Hg, Ag and Au (25, 26); the inhibition of phosphate absorption and of Mg^{++} absorption in yeast by arsenate (27); the actions of mercurials in reabsorptive systems in the kidney (28); and the action of Hg and Cu on the NaCl transport in frog skin (29, 30).

CONCLUSIONS AND SUMMARY

The studies that have been discussed here in detail, and others in the literature, illustrate some of the general problems involved in analyzing toxic effects at a molecular level in the cell. Knowledge of the sensitivities of individual enzyme systems, *in vitro*, does not allow a prediction of action of the metals on the cell. In the first place, the metals are not highly selective, but are capable of inhibiting a wide variety of enzymes. Secondly, the metal may never reach a particular enzyme site in the cell. Thus, the location of the metal sensitive sites in the cell is of greater importance than the inherent sensitivity of sites. For this reason, the membrane of the cell, which is directly accessible to the metal, is the first site of damage and sometimes the only important one. The cytoplasmic systems are protected in two ways: *a*) the membrane acts as a diffusion barrier and *b*) many physiologically inert substances in the cytoplasm react with the metal and reduce the amounts available to react with sensitive centers. Even within the cytoplasm, the arrangements of cellular systems may contribute to their metal sensitivity. Those systems closest to the membrane will tend to be most susceptible.

It is not always easy to differentiate cell membrane phenomena from those occurring within the interior of the cell. However, a number of useful criteria can be listed:

1) Reactions of heavy metals within the membrane are relatively rapid and reversible, whereas those occurring within the cell show a time lag and are sometimes essentially irreversible or very slowly reversible.

2) If known functions of the cell membrane are disturbed, then the metal is presumably acting on the membrane. Included in this category are *a*) inhibitions of enzymes located on the surface of the cell, *b*) inhibitions of specific transporting systems by which physiological substances such as sugars, amino acids, and ions are transferred into or out of the cells, *c*) breakdown of the membrane as a permeability barrier, and *d*) alterations of bioelectric potentials which in turn are related to ion transport and to permeability.

3) If the chemical interactions or the resultant physiological effects of the metal are responsive to extracellular rather than intracellular substances, then it may usually be presumed that the interaction is at the cell membrane.

In any study of toxicity of metals at a cellular level, two kinds of measurements can be made: *a*) the interaction of the metal with the cell in a chemical sense and *b*) the physiological disturbance. The chemical interactions can sometimes be characterized in some detail, based on known reactions of the metals with specific groups such as phosphoryl, carboxyl, imidazole, or sulfhydryl. Relating the interactions at this level to physiological effects is sometimes very difficult because the metals will combine with all accessible reactive ligands, whereas the response may be associated only with one type of ligand, or even with a small fraction of a given ligand. Some of the metal is thereby diverted away from sensitive sites.

On the physiological level, the responses may be of many types. It is of primary importance to study a response that is directly associated on the chemical insult. The longer the chain of events between the chemical insult and the measured response, the more difficult and complicated may be the analysis of the situation. If the metal acts on a specific chemical mechanism such as a surface enzyme, or a specific transport system, some of the properties or the system can be determined by a kinetic analysis, in much the same way as the inhibition of an enzyme can be analyzed. Some metals act as typical competitive inhibitors or non-competitive inhibitors of membrane function. If, on the other hand, the metal acts directly on the membrane structure leading to its breakdown, the response may be of the threshold type, fitting a population curve.

Despite the complexity of the biological system and its interactions with heavy metals, much useful information has been obtained concerning both the nature of the toxic effects and the parameters of membrane function.

REFERENCES

1. GURD, F. R. N. AND P. E. WILCOX. *Advance Protein Chem.* 11: 312, 1956.
2. SHACK, J., R. J. JENKINS AND J. M THOMPSELF. *J. Biol. Chem.* 198: 85, 1952.
3. DEMIS, D. J. AND A. ROTHSTEIN. *Am. J. Physiol.* 180: 566, 1955.
4. ROTHSTEIN, A., *The Enzymology of the Cell Surface, Protoplasmatologia E4*, Berlin: Springer, 1953.
5. ROTHSTEIN, A. AND R. MEIER. *J. Cell. & Comp. Physiol.* 34: 97, 1949.
6. ROTHSTEIN, A. AND C. LARRABEE. *J. Cell. & Comp. Physiol.* 32: 247, 1948.
7. ROTHSTEIN, A. AND A. D. HAYES. *Arch. Biochem.* 63: 87, 1956.
8. DOUNCE, A. L., G. H. TISHKOFF, P. FANTA AND T. H. LAN. In: *The Pharmacology and Toxicology of Uranium Compounds*, edited by Voegtlin and Hodge. New York: McGraw-Hill, 1949.
9. ROTHSTEIN, A. AND R. MEIER. *J. Cell. & Comp. Physiol.* 38: 245, 1951.
10. HIRAOKA, J. AND H. TAKADA. *J. Inst. Polytech., Osaka City Univ.* 7: 115, 1956.
11. ROTHSTEIN, A., R. MEIER AND L. HURWITZ. *J. Cell. & Comp. Physiol.* 37: 57, 1951.
12. BARRON, E. S. G., J. A. MUNTZ AND B. GASVODA. *J. Gen. Physiol.* 32: 163, 1948.
13. ROTHSTEIN, A., A. FRANKEL AND C. LARRABEE. *J. Cell. & Comp. Physiol.* 32: 261, 1948.
14. HURWITZ, L., Postdoctoral Thesis, University of Rochester, 1953.
15. ROTHSTEIN, A., *Symp. Soc. exp. Biol.* No. VIII: 165, 1954.
16. DEMIS, D. J., A. ROTHSTEIN AND R. MEIER. *Arch. Biochem.* 48: 55, 1954.
17. MYRBÄCK, KARL AND E. WILLSTAEDT. *Arkiv för kemi*, 8: 53, 1955.
18. ROTHSTEIN, A. In: *Electrolytes in Biological Systems*, edited by A. M. Shanes, Washington, D. C.: Am. Physiological Soc., 1955.
19. ROTHSTEIN, A. AND M. BRUCE. *J. Cell. & Comp. Physiol.* 51: 439, 1958.
20. PASSOW, H. AND A. ROTHSTEIN. *J. Gen. Physiol.* In press 1959.
21. PASSOW, H., A. ROTHSTEIN AND B. LOEWENSTEIN. *J. Gen. Physiol.* 43: 97, 1959.
22. ROTHSTEIN, A. *Radiat. Res. Suppl.*, New York: Acad. Press 1: 357, 1959.
23. JACOBS, M. H. *Ann. New York Acad. Sc.* 50: 824, 1950.
24. WILBRANDT, W. *Pflügers Arch. ges. Physiol.* 244: 637, 1941.
25. JOYCE, C. R. B., H. MOORE AND M. WEATHERALL. *Brit. J. Pharm.* 9: 463, 1954.
26. PASSOW, H. AND K. TILLMAN. *Pflügers Arch. ges. Physiol.* 262: 193, 1956.
27. JENNINGS, D. H., D. C. HOOPER AND A. ROTHSTEIN. *J. Gen. Physiol.* 41: 1019, 1958.
28. PITTS, R. F. *Am. J. Med.* 24: 745, 1958.
29. USSING, H. AND K. ZERHAN. *Acta physiol. scandinav.* 23: 110, 1951.
30. LINDERHOLM, H. *Acta physiol. scandinav.* 27: Suppl. 97, 1952.

DISCUSSION

T. W. CLARKSON

I SHOULD LIKE to extend the discussion to a consideration of the activity of metallic cations on epithelial tissue concerned with reabsorption processes such as occurs in frog skin, the kidney, and the gastrointestinal tract.

The problem which first presents itself is the question of which function of the tissue is most sensitive to damage by metals. It has been emphasized in the previous report that the cell membrane is the primary site of insult when suspensions of cells are exposed to solutions of metallic salts. The reaction proceeds rapidly. Quite low concentrations of metals produce structural and functional damage. As a result it is found that the membrane transport processes are the first of the cellular activities to be affected.

There is reason to expect that similar considerations apply to epithelial tissue. The 'active' cells responsible for the reabsorptive function of the tissue are arranged usually as a single layer and are in immediate contact with the surrounding media. At least some of the steps involved in the transport of substances across the tissue will be located in the cell membrane. In the case of sodium reabsorption in the kidney nephron, evidence has been presented by Cort and Kleinzeller (1) that the pumps responsible for the active transport of sodium out of the cell are also involved in the net transport of sodium across the tissue. For these reasons it may be anticipated that metals will effect epithelia tissue similarly to cell suspensions and that the transport processes across the tissue will provide the most sensitive measure of injury.

There are, however, certain obvious differences between epithelial tissue and single cell suspensions. In the first place, besides the 'active' cells, other cell types may be present. Secondly, there is also present an intercellular cement material holding together the various cells. As a result, a metal may be bound by epithelial tissue at many different loci not all of which will involve the 'active' cells. Greater amounts of metal will be required to produce inhibition of the cellular transport processes and a wider variety of responses may be expected than is the case with single cell suspension.

The intercellular cement may be an important structure with regard to some of the transport processes. Water reabsorption in the distal tubule and collecting ducts of the mammalian kidney occurs intercellularly and indeed is said to be controlled by the degree of polymerization of the hyaluronic acid of which the cement is chiefly composed (2). Intercellular cements consist mainly of acidic polysaccharide whose various anionic groups are a likely point of attack by metals.

To illustrate these points, I should like to discuss two kinds of studies: one carried out on the isolated tissue where quantitatively accurate measurements have been made under carefully controlled conditions; the other involving studies on human subjects which have useful application in the field of industrial toxicology.

ACTION OF HG^{++} AND CU^{++} ON FROG SKIN

Such tissue as frog skin and gastro-intestinal epithelia may be isolated from the animal as a sheet and held tightly between two chambers containing solutions of any desired composition. The tissue remains active for many hours transporting solutes from one compartment to the other. Such solute movements are in general classified as 'active' or 'passive' according to whether or not the solute is transported against a net energy gradient. These two processes may be distinguished and accurately measured (3, 4).

When frog skin is placed in this system, sodium chloride is transported from the outside to the inside compartment—that is, in the physiological direction. This is due to an active absorption of sodium ions followed by the passive movement of chloride ions to maintain electro-neutrality. As a result, the inside of the skin is 40 mv positive with respect to the outside. When the sodium pump becomes inactive, the potential falls to zero. When chloride is replaced by an impermeable anion such as iodide or sulfate the potential rises since the electrical resistance of the membrane has increased.

Linderholm (4) studied the effect of the organic mercurial, mersalyl and mercuric chloride on an isolated preparation of frog skin separating two Ringers solutions. When the outside solution was made 10^{-4} M with respect to mercury, the potential rapidly fell to zero. Linderholm was able to demonstrate that this was due to a complete blockage of the active transport of sodium ion, there being no detectable effect on the passive penetration of Cl^-. The same concentration of mersalyl caused, if anything, a slight increase in the respiration of the tissue.

When a 10^{-5} M $CuSO_4$ solution is in contact with the outside of the frog skin, a completely different response develops (3). The skin potential rises from a control value of about 24 mv to values of the order of 90 mv. Ussing has attributed this to a specific blockage of the chloride pathway. There is no detectable effect on the activity of the sodium pump.

It is of interest to compare the action of the two metals. Both mercury and copper behave uniquely and in a specific fashion at low concentrations of the metal are required to inhibit the metabolism of the cells. Following the arguments presented by Dr. Rothstein, when he considered the action of mercury and copper on muscle, it would appear that these metals are inhibiting the transporting activities of the tissue through an interaction with essential components on the surface of the 'active cells'. Only when the concentrations of the metals are greatly increased, do sufficient amounts penetrate to the interior of the cells and inhibit the respiratory systems.

It is obvious that, arising from their specific effects, both Hg^{++} and Cu^{++} are useful biochemical tools in studying reabsorptive processes. Indeed $CuSO_4$ may be used to produce an anion impermeable frog skin, thereby allowing measurements to be made of the extent to which each side of the frog skin is permeable to sodium, potassium, and other cations (5).

The possibility has been considered (4, 5) that certain solutes may penetrate frog skin via holes in the intercellular cement, rather than directly through the 'active cells'. Such a pathway would seem applicable to those solutes such as chloride that do not require for their transport a supply of metabolic energy from the cells. If it could be demonstrated that the cupric ions were specifically bound by the intercellular cement, then this would support the idea that the passive penetration of chloride occurred through these structures.

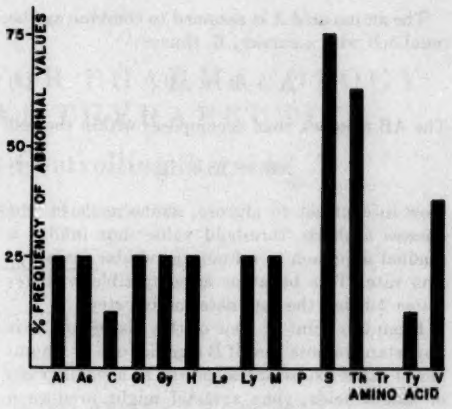


FIG. 1. Percentage frequency of abnormally high excretion (P = .003) for urinary amino acids of workmen under slight exposure to cadmium oxide fume. These men did not exhibit clinical symptoms of cadmium toxicity. Al = alanine; As = aspartic acid; C = cystine; Gl = glutamine; Gy = glycine; H = histidine; Le = leucine; Ly = lysine; M = methyl histidine; P = proline; S = serine; Th = threonine; Tr = tyrosine; V = valine.

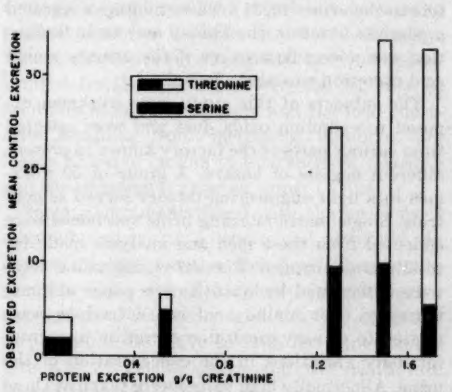


FIG. 2. Relation between the proteinuria resulting from exposure to cadmium oxide fume and the urinary excretion of threonine and serine. Protein values serve as a rough index of kidney damage.

ACTION OF CD^{++} ON THE HUMAN KIDNEY—IN VIVO

It is also possible to study the action of metals on the transport processes of epithelial tissue in the living animal. This type of work presents new problems. Experimental conditions are no longer under the same rigid control as in the case of isolated preparations. Often the experimenter is unable to measure all the relevant parameters. Consequently the observations are subjected to statistical analysis.

Nevertheless, despite these difficulties, there are examples in the literature in which it has been found that metals can produce an inhibition of the transport processes just as specific as those already discussed for isolated epithelia. An excellent case in point is the action of cadmium on the reabsorption of amino acids by the human kidney.

Amino acids are handled by the kidney in a similar manner to glucose (6). The plasma amino acids after passing into the glomerular filtrate are reabsorbed in the proximal portion of the kidney tubule. Normally the reabsorption process is quite efficient, returning some 98–99% of the filtered amino acids back to the blood stream. However, if a particular amino acid is presented to the proximal tubule at increasing concentrations, it is found that the reabsorption process gradually approaches a maximal limiting rate. For this reason, it has been suggested (7) that amino acids are transported across the kidney epithelium by a carrier system somewhat similar to that proposed for glucose reabsorption by Shannon and Fisher (8).

The amino acid A is assumed to combine on the renal cell with a carrier, B, thus:—

$$A + B \rightarrow AB \quad (1)$$

The AB complex then decomposes within the cell

$$AB \rightarrow A + B \quad (2)$$

Now in contrast to glucose, amino acids do not possess a sharp 'threshold value' but inhibit a gradual approach to a limiting tubular reabsorption rate. This behavior is compatible with *reaction 1* being the rate-determining step.

From the point of view of this discussion, it is important to note that if B signifies one of a number of carrier substances specific to a small group of amino acids, then a metal might produce a specific response by interacting a particular carrier and thereby affecting the reabsorption of only a limiting group of amino acids. A report by Clarkson and Kench (9) on the *in vivo* action of cadmium on the human kidney illustrates that this might well occur.

It had been known for some time that the prolonged exposure of workmen to cadmium oxide fumes led in many cases to an appearance of protein in the urine (10, 11). These findings suggested a possible lesion in the kidney and an investigation was undertaken to see if the urinary amino acid excretion was also affected.

The subjects of this study were workmen exposed to cadmium oxide dust and were selected from various parts of the factory known to present different degrees of hazard. A group of 50 workmen in a light engineering factory served as controls. Single, early morning urine specimens were collected from these men and analyses made for total amino-nitrogen. The individual amino acids were determined by quantitative paper chromatography. The amino acid concentrations were related to urinary creatinine excretion to correct for daily variations in the concentration of the urine. Abnormally high values were taken as those differing from the mean of the controls by more than three standard deviations ($P = .003$). The excretion of cadmium and protein were taken as an index of exposure. None of the workmen exhibited clinical symptoms of poisoning.

There are two points arising from this data, which are of interest to the present discussion. In the first place, in view of the observed proteinuria and the findings of Friberg (10) of decreased renal efficiency, it seems most probable that cadmium is acting on the kidney epithelia to produce inhibition of those processes responsible for the reabsorption of amino acids. If this assumption is correct, the data lend support to the concept of differing pathways of reabsorption of amino acids suggested by the work of Pitts (12) and Beyer, Sheggs, Russo and Shaner (13)—the particular conclusion in this case being that there is one pathway through which the hydroxy-amino acids are reabsorbed and that this is specifically blocked by cadmium.

The second point of importance is that this kind of specific biochemical lesion, once detected, may be of use not only to the research worker interested in the mechanism of renal transport processes but also to those responsible for the control of metal poisoning in industry, in that it may provide an invaluable diagnostic tool. In this respect it is pertinent to note that abnormal threonine and serine excretion was detected at a stage of cadmium exposure prior to the appearance of the clinical poisoning syndrome. Such observations could form the basis of a presymptomatic test. This is particularly important in cadmium poisoning where damage to lung tissue is cumulative and irreversible.

REFERENCES

1. Cort, J. H. and A. Kleinzeller. *J. Physiol.* 142: 208, 1958.
2. Ginetzinsky, A. G. *Nature, London* 182: 1218, 1958.
3. Ussing, H. H. and K. Zerahn. *Acta physiol. scandinav.* 23: 110, 1951.
4. Linderholm, H. *Acta physiol. scandinav.* 27 - Suppl. 97, 1952.
5. Ussing, H. H., IV Internat. Congress of Biochem., Vienna, 1958.
6. Smith, H. W., *The Kidney*, (1st ed.), New York: Oxford, 1951.
7. Pitts, R. F., *Am. J. Physiol.* 140: 156, 1943.
8. Shannon, J. A. and S. Fisher. *Am. J. Physiol.* 122: 765, 1938.
9. Clarkson, T. W. and J. E. Kench. *Biochem. J.* 62: 361, 1956.
10. Friberg, L., Arch. industr. Hyg. 4: 458, 1950.
11. Smith, J. C., J. E. Kench and R. E. Lane. *Biochem. J.* 61: 698, 1955.
12. Pitts, R. F. *Am. J. Physiol.* 140: 535, 1944.
13. Beyer, K. H., L. D. Wright, H. F. Skeggs, H. F. Russo and G. A. Shaner. *Am. J. Physiol.* 151: 202, 1947.

AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

Symposium on Fertility-Controlling Steroids[1]

Chairman: HUDSON HOAGLAND

INTRODUCTION

HUDSON HOAGLAND

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

WE HAVE A BIBLICAL PROPHECY to justify the holding of Federation symposia of this kind in which many contributors from far-flung institutions come together to discuss their work. I learned from a recent annual report of the Carnegie Corporation that in Chapter 12 of the Book of Daniel it says: "Many shall run to and fro and knowledge shall be increased."

Within the past decade certain of the 19-nor steroids have proved of value in treating menstrual disorders; and the actions of these and related progestational compounds on human fertility have been investigated during the past four years. The finding that some of these steroids block ovulation without untoward side effects has been of special interest to those concerned with the current explosive increase in population throughout the world. Population increases are especially pronounced in economically unfavored countries and are, in the main, a result of the recent introduction of public health measures including the use of antibiotics and other medical advances that have dramatically increased life expectancy and reduced death rates, while birth rates have held constant or increased with improvements in health. At its present rate of growth, which is increasing, the world population is more than doubling every forty years and various demographers predict a population of from 9 to 13 billion a century hence compared to that of 2.8 billion today. The prospects of supplies of food, of energy and other economic resources to keep pace with such prospects are grim, to say the least.

While the populations of Europe and the United States have an increasing number of elderly people resulting from several generations of medical advances, the underdeveloped countries where population growth is most marked contain ⅓ of the world's people and have, on the average, a much younger population because of marked postwar declines in infant mortality. The inadequacies of educational and job opportunities for these young people, the inability to accumulate capital resources and the food shortages resulting from increasing numbers, are highly abrasive factors in international relations and the situation is growing worse.

Some governments faced with the problem of over-population have come out strongly in support of contraceptive measures. Thus Japan, a country the size of Montana, in 1920 had a population of 51 million and this had increased to 91 million by 1957. In 1948 the annual birth rate was 34 per thousand and the death rate 12 per thousand. This would have doubled the population in 32 years. As a result, in 1948 the Japanese Diet took the drastic step to legalize abortion and established 30,000 birth-control guidance offices. As a consequence the birth rate fell to 19.4 in 1955 cutting the rate of growth almost in half and bringing it below that of the United States. India has built into its second 5-year plan provisions for research in population control. The province of Kerala has offered approximately five dollars to each of its male citizens who submit to vasectomy. On February 16, 1959 the New York Times reported that the Indian government has decided to provide facilities in all hospitals to carry out sterilization

[1] Atlantic City, New Jersey, April 14, 1959.

operations for all who wished to restrict the size of their families. The Indian Ambassador to the United States said in a television interview last winter that one of the most pressing needs facing his country was a practical, safe and cheap oral contraceptive.

Investigations of the use of 19-nor steroids as oral contraceptives are discussed in several papers of this symposium. Whether they or related substances may ultimately come to play a significant role in population control remains for the future to determine. While interest in the contraceptive properties of the progestins discussed in the papers of this symposium is considerable, it should be borne in mind that studies of these substances have advanced our fundamental knowledge of the processes of mammalian reproduction. These basic advances are of much value quite aside from clinical or other practical considerations of the role that these substances may play.

BIOLOGICAL EFFECTS OF SOME STEROIDS WITH PROGESTATIONAL ACTIVITY

VICTOR A. DRILL

Division of Biological Research, G. D. Searle & Co., Chicago, Illinois

PROGESTERONE IS concerned with two important functions: reproduction and development of secondary sex characteristics. Our attention at this symposium will be directed chiefly at the relationship of newer progestational substances to reproductive problems. These newer progestational compounds have wide application in the treatment of functional bleedings, amenorrhea and infertility problems. Further, several of the newer compounds, when administered at certain times during the menstrual cycle, can inhibit ovulation and thus serve as antifertility or contraceptive agents.

Progesterone is poorly effective orally and is usually administered parenterally. Ethynyl testosterone, a compound developed in 1938, is progestational and active orally (1). Oral activity is, however, comparatively low and androgenic side effects may be produced (2). Many earlier attempts to modify the structure of progesterone and increase potency and oral effectiveness were unsuccessful, but in the last few years interest in this area has revived and a number of potent progestins, effective orally, are now known (3, 4). That these compounds have shown hitherto unexplored endocrine effects and mixtures of biological properties has only added to their interest.

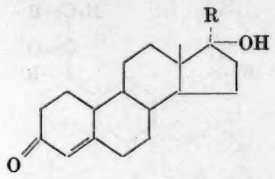
The present brief review will be concerned with: *1*) structure and progestational activity, *2*) other endocrine properties of the progestational compounds, *3*) antifertility activity and *4*) side effects and safety.

CHEMICAL STRUCTURE AND PROGESTATIONAL ACTIVITY

Progestational activity may be determined by administering compounds to estrogen-primed rabbits and grading the histological change produced. In the Clauberg method (5) substances are administered orally or parenterally to animals previously injected with estrogen. In the McGinty technique (6) microgram amounts of the test material are injected into the uterine horn of the estrogen-primed rabbit. Thus this latter test measures the local or direct effect of the administered compound. Recently Lutwak-Mann and Adams (7) have demonstrated that the carbonic anhydrase activity of the endometrium can be used as an index of progestational proliferation in the rabbit. This method may also be used to determine progestational activity of new steroids, and estimates of potency agree well with determinations made in the Clauberg rabbit (8–10).

With reference to tables 1–5 below, progesterone was assigned a potency of 100% and used as the standard in the McGinty and subcutaneous Clauberg tests. Inasmuch as progesterone is poorly effective orally, it could not be administered by this route. Therefore, in studying the oral activity of new steroids the minimal effective

TABLE 1. PROGESTATIONAL ACTIVITY OF 19-NORTESTOSTERONE DERIVATIVES



R Substituent	McGinty Test	Clauberg Test	
		Subcutaneous	Oral
	$\Delta^{4(5)}$ Derivatives		
	%	%	%
H	0*	<5	<20
Methyl	0	500	100
Ethyl	25	500	250
Vinyl	0	150	250
Ethynyl	0	50	100
Propyl	500	250	25
Butyl	1000	100	<25
Octyl	50	10	
	4,5-Dihydro derivatives		
Methyl	0	10	<10
Ethyl	50	25	10
Propyl	0	<5	

* = <0.5%.

dose is stated in terms of the minimal effective dose of progesterone administered subcutaneously. Thus, a compound listed in the tables as having 100% activity is as active orally as is progesterone administered subcutaneously.

19-Nor-steroids. Allen and Ehrenstein (11) first reported that 19-norprogesterone was active progestationally. Later Tullner and associates (12) found the compound to be about five times as effective as progesterone in the Clauberg rabbit assay. (In 19-nor compounds the methyl group at position 10 of the steroid molecule is removed.) Hertz *et al.* (13, 14) also observed the 19-nor derivative of 17-ethynyl testosterone to be approximately five times as potent as the parent compound. It is also active orally in women (14–16). Another compound, 17-ethyl-19-nortestosterone, was noted by ourselves (17) and by Pincus and associates (18) to be more active than progesterone on injection and more potent orally than norprogesterone. Pincus *et al.* (18) also reported on the progestational activity of the methyl and ethynyl derivatives. The activity of these 19-nor compounds then led to the detailed exploration of related structures.

19-Nortestosterone Derivatives. We havs studied a series of 17-derivatives of nortestoe-

TABLE 2. 17-ALKENYL DERIVATIVES OF 19-NORTESTOSTERONE

17 Substituent	McGinty Test, %	Clauberg Test	
		Subcutaneous %	Oral %
$-CH=CH_2$	0*	500	250
$-CH_2-CH=CH_2$	50	500	250
$-CH(CH_3)-CH=CH_2$	1000	500	100
$-CH_2-CH(CH_3)=CH_2$	1000	1200	300
$-C\equiv CH$	0	50	100
$-C\equiv C-CH_3$	0	500	200
$-C\equiv C-C_2H_5$	5–50	200	100
$-C\equiv C-C_4H_9$	500	100	100

* = <0.5%.

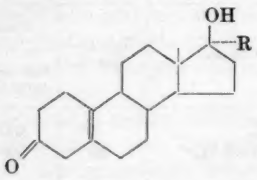
terone in which the side chain varied from H to C_8H_{17} and observed that activity was related to the degree of saturation, the length of the 17-substituents side chain, and the presence or absence of a 4(5) double bond (19, 20).

The alkyl derivatives reach peak activity in the McGinty test with a butyl side chain, whereas in the Clauberg test greatest potency is obtained with the methyl and ethyl derivatives (table 1). Overbeek has also reported the methyl derivative to be very active in rabbits (21), and McGinty and Djerassi, in general agreement with the above reports, find the methyl, ethyl, vinyl and ethynyl analogues to have similar potency (22). The methyl, ethyl, and ethynyl derivatives are active orally in women as progestins in doses of 10 and 20 mg (4, 23). It will be noted that when the 4(5) double bond is saturated activity markedly decreases.

Progestational activity is also related to route of administration. Some compounds were not active when applied locally in the McGinty test but did show progestational effects in the Clauberg test following subcutaneous or oral administration. These results will be discussed later in regard to maintenance of pregnancy.

It is apparent that the higher homologues of 19-nortestosterone show an increase in progestational activity as measured by the McGinty or Clauberg test. In view of this result, the effect of homologous derivatives of 17-ethynyl and 17-vinyl-19-nortestosterone was investigated (table 2). Of the alkenyl derivatives, the 17-allyl and 17-(2-methallyl) substituted compounds

TABLE 3. PROGESTATIONAL ACTIVITY OF SOME 5(10)-ESTRENOLONE DERIVATIVES



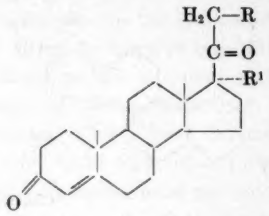
Substituent	McGinty Test	Clauberg Test: Subcutaneous	Clauberg Test: Oral
	%	%	%
Methyl	0	5	25
Ethyl	25–50	10–25	25 or >
Vinyl	0	25	
Ethynyl	0	0–25	25
Propyl	25	50	10

showed good activity in these tests. In women, however, the 17-(2-methallyl) derivative has not been as active as the lower alkyl derivatives. The 21-methyl derivative of ethynyl-19-nortestosterone, because of its high activity and other considerations of related endocrine properties, is presently being evaluated clinically.

Ethynyl Testosterone Derivatives. The activity of ethynyl testosterone may be increased by substituting alkyl groups in place of the 21-hydrogen. Such studies, performed by Hartley and co-workers (24) show that the methyl derivative is three times as effective orally as ethynyl testosterone. 17-Propargyl testosterone has been prepared earlier (25, 26) and is reported to have minimal activity (25).

$\Delta^{5(10)}$ Estrenolones. The effect of 17-alkyl and alkynyl substituents on progestational activity when the double bond is shifted from the 4(5) to the 5(10) position was explored. Pincus *et al.* (18, 27) have earlier reported on the progestational activity of the ethynyl derivative. It is evident from table 3 that the activity of such compounds in the Clauberg test is not as great as analogues in the nortestosterone series (28), whereas Pincus *et al.* (10) find the ethynyl-5(10)-17-OH-estrenolone to have the same progestational potency as 17-ethynyl-19-nortestosterone. However, the pure 17-ethynyl derivative (norethynodrel) possesses inherent estrogenicity and it is known that the standard estrogens can decrease or block a progestational response in the Clauberg test. This appears to be the case, for when norethynodrel was studied in women it was observed to be a potent compound, being

TABLE 4. 21-HALOGENATION OF PROGESTERONE AND 17-OH-PROGESTERONE ACETATE*



R	R¹	Clauberg Test: McGinty Test	Clauberg Test: Subcutaneous	Clauberg Test: Oral
		%	%	%
Cl	H	5	0	
F	H	500	200	5
Br	OAc	500	100	5
Cl	OAc	1000	200	<20
F	OAc	100	1000	100

* Data from Elton (41).

effective orally in a dose of 10 or 20 mg per day (4, 29, 30).

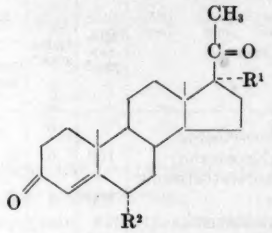
9-Halogenated Progesterones. Fried, Kessler and Borman (31) have studied the effect of 9-halogenation and the 11β-OH and 11-keto group on the activity of progesterone. Such substituents decrease the parenteral activity, but oral potency is greater than that of ethynyl testosterone. The data of Wied and Davis (32) indicate that 9α-Br-11-ketoprogesterone is active orally as a progestin in women but that potency is less than that of the nortestosterones and $\Delta^{5(10)}$ estrenolones discussed above.

12-Halogenated Progesterones. The effect of 12α-halogenation on progestational activity has also been studied (31). Substitution in this position has less of an effect on potency and, also, association with an 11β-OH group is important as contrasted with an 11-keto group.

11β-Hydroxy Derivatives. The introduction of an 11β-OH group has been observed to decrease progestational activity in compounds of the natural progesterone series (33). The introduction of this group into 17-ethynyl-19-nortestosterone practically eliminates progestational activity. However, the 11β-OH group can exert some effect in 12-halogenated steroids.

10β-Hydroxy Derivatives. The introduction of a 10β-OH group into 17-ethynyl-19-nortestosterone was reported by Djerassi and co-workers to decrease progestational activity; potency was less than 25% that of ethynyl-19-nortestosterone (34).

TABLE 5. 6-METHYL DERIVATIVES OF PROGESTERONE*



17 Substitution R^1	6 Substitution R^2	McGinty Test	Clauberg Test	
			Subcutaneous	Oral
		%	%	%
OH		0	0	0
OAc		100	1000	20
	CH_3	500	800	20
OAc	CH_3	2500	3000	500

* Data from Elton (41).

17-Hydroxyprogesterone and Derivatives. The progestational activity of the 17α-OH derivative is low, being about 1/100 the activity of progesterone (35). However, esterification to form the acetate or caproate endows the molecule both with progestational activity and a prolonged duration of action (36, 37). Oral potency is very low. Esterification of 17-ethynyl-19-nortestosterone to produce 17-acetate or 17-enanthate also produces a long-acting compound on parenteral administration (32, 38). In our own studies, esterification of 17-propynyl-19-nortestosterone and related compounds also increases activity. Junkmann and Witzel have reviewed the chemistry and pharmacological properties of the steroid-hormone esters (39, 40).

21-Halogenated Progesterones. Elton (41) has studied the effect of the 21-halogenation of progesterone and 17-OH progesterone acetate (table 4). Activity, although high in the McGinty test, decreases in the Clauberg test.

6-Methylation. Hartley and co-workers (24) studied the effect of 6-methylation on the activity of ethynyl testosterone. The 6α-methyl but not the 6β-methyl derivative showed an increased progestational potency. Campbell *et al.* (42) report 6α-methyl-17-ethynyl testosterone to have only twice the activity of ethynyl testosterone. Hartley *et al.* (24) obtained greatest activity when ethynyl testosterone was methylated in both the 6α and 21 positions. Data from our laboratory indicate that the potency of the 6,21-dimethyl derivative is comparable in the oral Clauberg assay to that of ethynyl testosterone.

TABLE 6. PROGESTATIONAL ACTIVITY AND MAINTENANCE OF PREGNANCY*

Compound	Assay		Pregnancy	
	Mc-Ginty	Clau-berg	Rat	Rabbit
Progesterone	+	+	+	+
19-Nortestosterones				
17-Methyl	0	+		0
17-Ethyl	+	+	+	+
17-Ethynyl	0	+	0	0
17-Propyl	+	+	+	+
17-Allyl	+	+	+	+
17-OH-$\Delta^{5(10)}$ Estrenolones				
17-Ethynyl	0	+	0	0

* Data from Saunders and Elton (49).

Very high progestational potency has been found by Babcock and co-workers to occur when the 17-OH-acetate of 6α-methylprogesterone is prepared (43). This high activity has been confirmed by Miyake and Pincus (10) and by ourselves (41). The data in table 5 demonstrate the high activity in the Clauberg test following subcutaneous administration. Potency decreases when the compound is given orally. Greenblatt has shown that the compound is active clinically (44).

The 6α-fluoro derivative of progesterone has been prepared by Hogg *et al.* (45) and by Bowers and Ringold (46). Hogg and associates state that the derivative has 6 to 10 times the oral potency of progesterone. They also prepared the 6α-fluoro derivative of ethynyl testosterone but did not give its biological potency.

Dehydro Compounds. 6-Dehydroprogesterone is reported to have one-half the activity of progesterone. However, the 6-double bond will increase progestational activity of 17-alkynyl-19-nortestosterone derivatives. The reverse situation appears to be present when an 11,12-double bond is introduced. This bond has been reported to increase the activity of progesterone but appears to decrease the activity of 17-ethynyl-testosterone (47).

OTHER ENDOCRINE PROPERTIES OF PROGESTATIONAL COMPOUNDS

Maintenance of Pregnancy. When pregnant rats or rabbits are ovariectomized on the eighth and twelfth days, respectively, of pregnancy, the

TABLE 7. COMPARATIVE ENDOCRINE ACTIVITIES OF 17-SUBSTITUTED 19-NORTESTOSTERONE DERIVATIVES

17 Substituent	Androgenic Potency*	Anabolic Potency*	Progestational Potency†	
			McGinty test	Clauberg, s.c.
	%	%	%	%
H	6	100	0	<5
Methyl	6	100	0	500
Ethyl	6	100	25	1000
Propyl	<1	10	500	250
Vinyl	2	20	0	500
Ethynyl	2	1	0	50
Allyl	1–5	10	50	500
1-Methallyl	1	5	1000	500
2-Methallyl	1	<5	1000	1200

* Testosterone propionate = 100% in anabolic and androgenic assays.

† Progesterone = 100% in progestational assays.

fetus will be resorbed. Injections of progesterone will maintain pregnancy. When some of the newer potent, progestational compounds were studied in this regard, it was observed that not all of them maintain pregnancy (table 6). Comparison with the progestational tests indicates that only compounds active in the McGinty test maintain pregnancy in rats and rabbits (48, 49). The fact that compounds are active following subcutaneous or oral administration in the Clauberg test does not necessarily indicate that they will maintain pregnancy. Thus, endometrial response and effect in pregnancy appear to be separable effects. Pincus *et al.* (28) have also reported that Norlutin and Enovid do not give a decidual response in rats and do not maintain pregnancy in rabbits, whereas progesterone and Nilevar are active in both types of studies. 17α-Hydroxyprogesterone-17-caproate (Delalutin) does not maintain pregnancy in mice (50).

Myotrophic Activity. Myotrophic activity, as determined by the increase in weight of the levator ani muscle, is independent of the progestational activity of active steroids (table 7).

Androgenic Activity. Androgenic activity is compared with myotrophic activity in table 7 and with estrogenic activity in table 8. The compounds listed in table 8 are being employed clinically. The nortestosterone series retains minimal androgenicity, as measured by the increase in ventral prostate and seminal vesicle weight in castrated male rats. The estrenolone derivative has, on the other hand, estrogenic

TABLE 8. ANDROGENIC, MYOTROPHIC AND ESTROGENIC ACTIVITY

Compound	Myotrophic*	Androgenic*	Estrogenic†	
			Uterine weight	Vaginal smear
17-Methyl-nortestosterone (Neosteron)	100	6	0.2	0
17-Ethyl-nortestosterone (Nilevar)	100	6	0.3	0
17-Ethynyl-nortestosterone (Norlutin)	1	2	0.01	0
17-Ethynyl-$\Delta^{5(10)}$ estrenolone (Enovid)	0	0	7	3–5

* Testosterone propionate = 100% in myotrophic and androgenic assays.

† Estrone = 100% in estrogenic assays.

rather than androgenic activity. The findings in man agree fairly well with the experimental data. In man, 17-methyl-19-nortestosterone is the most androgenic of the group and produces androgenic side effects (51). 17-Ethyl-19-nortestosterone is devoid of androgenic effects at doses of 20–30 mg per day. Minimal androgenicity may be observed in some patients at doses of 50 mg a day, the effect becoming greater at doses of 75 or 100 mg a day. 17-Ethynyl-19-nortestosterone may also produce androgenic side effects (29). The estrenolone compound, Enovid, does not produce any signs of androgenicity in man, even when administered in high doses over a long period of time in the treatment of endometriosis (29, 52, 53).

Estrogenicity. Derivatives of nortestosterone produce a very slight increase in the uterine weight of mice. This small effect, observed at high dose levels, is characteristic of a progestin and does not indicate estrogenicity. In vaginal smear studies on rats, which is a more selective test for estrogenicity, such compounds are inactive (table 8). It is particularly important that completely pure samples of steroids be used in studies of estrogenicity as a small amount of contaminating steroid may give a positive response (22). 17-Ethynyl-$\Delta^{5(10)}$ estrenolone, on the other hand, has estrogenicity equivalent to 3 to 5% the potency of estrone (table 8). (Enovid contains 17-ethynyl-$\Delta^{5(10)}$estrenolone plus 1.5% of 3-methyl ether of ethynylestradiol. The data in table 8 refer to the pure estrenolone.) Such activity was obtained with pure compound after exhaustive recrystallization and chromatography and is not due to any impurity. This steroid is the

TABLE 9. QUALITATIVE COMPARISON OF ENDOCRINE PROPERTIES OF NEW PROGESTATIONAL COMPOUNDS

Compound	Prog.	Andro-genic	Ana-bolic	Estro-genic	Anti-Estr.	Pit. Inhib.
Neosteron	+	+	+	0	+	+
Nilevar	+	+	+	0	+	+
Norlutin	+	+	+	0	+	+
Enovid	+	0	0	+	0	+

first to possess both progestational and estrogenic activity in the same molecule.

Anti-estrogenic Activity. An anti-estrogenic compound will block the increase in uterine weight produced by estrone. All of the 17-alkyl derivatives of 19-nortestosterone possess anti-estrogenic activity. This property is not related to androgenic activity. Based on the minimal androgenic potency of the compounds, they are much more potent than testosterone as anti-estrogens. Thus, the anti-estrogenic effect of these compounds appears to be an independent property of the molecules. Neosterone, Nilevar and Norlutin are potent anti-estrogenic compounds (54). Norethynodrel has been studied in this regard and, although it does not appear to block the effect of estrone, any anti-estrogenic effect may be masked by the estrogenicity of the compound.

Pituitary Inhibition. Saunders (48) has studied the effect of Nilevar and norethynodrel on pituitary gonadotrophins. Donor rats received 1 mg of steroid daily for 5 days. The pituitary gland was removed, homogenized, and injected into test rats over a period of 3 days. The pituitary gland from untreated control animals produces a marked increase in ovarian weight in the test rats. The pituitary gland from Nilevar-treated rats produced an ovarian response equivalent to that observed from untreated animals, whereas the pituitary from norethynodrel-treated animals produced a much smaller increase in ovarian weight.

Epstein, Kupperman and Cutler (29) studied the ability of these steroids to inhibit pituitary gonadotrophin in parabiotic rats as judged by the ovarian weight response. They concluded that Enovid and Norlutin were potent inhibitors of pituitary function.

Possible Interconversion of Norethynodrel and Norethyndrone. These two compounds differ structurally only in the placement of the double bond, and several studies have been made to determine whether or not interconversion occurs. Ringold (55) has pointed out that some isomerization occurs at acid and alkaline pH, although the conditions required are more vigorous to shift a $\Delta^{5(10)}$ bond than to shift a $\Delta^{5(6)}$ to the $\Delta^{4(5)}$ position. When injected into mice, and effect on uterine weight determined, Edgren (54) has shown that norethynodrel produces a steep dose-response characteristic of an estrogen, whereas norethyndrone produces a shallow dose-response curve of a progestin. When administered orally, each compound produces its characteristic response curve demonstrating that at least in the mouse oral administration is not followed by the metabolism of one compound to the other (54). As discussed above under anti-estrogenicity, norethyndrone is anti-estrogenic, whereas norethynodrel, administered parenterally or orally under the same conditions, does not produce a similar response. Further, norethyndrone may produce minimal androgenicity in some patients, whereas this has not been observed following the administration of norethynodrel for prolonged periods of time. Although such studies do not rule out minimal interconversion, the experimental and clinical observations demonstrate that biologically these steroids are distinct entities.

Summary of Endocrine Properties. The different endocrine properties possessed by these new steroids have been discussed from a quantitative point of view. The comparative qualitative properties of the most interesting of these new progestational steroids that can affect fertility are summarized in table 9.

ANTIFERTILITY ACTIVITY

Antiovulatory Activity. It was first noted by Pincus and associates (18, 28) that Nilevar, Norlutin and Enovid would inhibit ovulation in the rabbit. Nilevar was the least potent of these three compounds although all were more active than progesterone. They also demonstrated that following oral administration these compounds reduced fertility in rats. Rock, Garcia and Pincus (56–58) then demonstrated that these compounds can inhibit ovulation in normal ovulating women as judged by blockage of the normal rise in basal temperature and a decrease in pregnanediol excretion.

Some studies have attempted to define the site of action of such compounds. It is known, for example, that Gonadophysin injections markedly increase ovarian weight, and Saunders (48) observed that the effect on the ovary was not blocked by norethynodrel. It is probable that

these steroids act to block ovulation not by affecting the ovarian response to gonadotrophins, but by inhibiting the formation or release of pituitary gonadotrophins, as discussed above.

Antifertility Activity. Steroids formed in the body, such as testosterone, progesterone, or estrone, can inhibit ovulation in women. Practically, however, they are not of value as antifertility or contraceptive agents in women. For example, testosterone produces undesirable androgenic effects, progesterone requires very large doses and its action is too variable, and estrogens, although they will inhibit ovulation in the first cycle, will not usually do so in the succeeding cycle. In contrast, Rock and co-workers observed that norethynodrel can inhibit ovulation in succeeding cycles (56–58).

Saunders (48) compared the effects of norethynodrel, progesterone, estrone and testosterone on fertility in rats. The compounds were administered for 35 days, and the dose of each compound necessary to prevent fertility determined. At the end of the treatment period rats in each group were autopsied and ovarian inhibition was observed, as demonstrated by the effect on ovarian weight. Treatment was then discontinued and the rats observed for future fertility. After 35 days of the recovery period, ovarian weight of the norethynodrel-treated animals had returned to the normal control range. During the recovery the treated animals became pregnant and in most treated groups the per cent pregnancy approached that of the control group. Recovery was complete in the norethynodrel-treated rats.

Rock, Garcia and Pincus have also demonstrated that the daily administration of norethynodrel, starting early in the cycle, continually blocks ovulation (56–59). Spotting may occur in the subjects so that they added 1.5% of an estrogen, 3-methyl ether of ethynylestradiol, to control this factor. Norethynodrel with 1.5% of this estrogen is Enovid. Extensive clinical studies on the antiovulatory activty of Enovid in over 800 women have been performed by Pincus (59) and will be reported later this afternoon.

Norethynodrel (Enovid) is the most potent of the steroids in blocking ovulation in normal women, being effective in doses of 5 mg and even 2.5 mg per day. Activity in probably due to the inhibition of pituitary gonadotrophins (60). However, activity is not simply related to progestational potency, for the potent progestin 6α-methyl-17-OH-progesterone acetate appears to be inadequate for this purpose even in doses of 30 or 40 mg a day. Pure norethynodrel is effective in low doses in blocking ovulation. Although the pituitary gonadotrophins are blocked, norethynodrel acts directly to cause growth of the endometrium, so that when treatment is stopped on the 25th day, withdrawal bleeding occurs from the anovulatory cycle. The spotting that occurs in some patients during treatment can be controlled by added estrogen, as is done with Enovid. The withdrawal bleeding that occurs each month is psychically important to the patient, and indicates further to the subject that she is not pregnant.

SAFETY

Side Effects and Safety. All of the newer progestational steroids produce some nausea and in certain groups of patients the incidence may be higher with Enovid. However, the overall incidence is close to the placebo range and, in the contraceptive studies of Pincus, may not be different from placebo control patients (59). Further, the patients' reports of nausea are significantly less after the second and third month of cyclic therapy. In such patients the cyclic therapy with Enovid produces an endometrial change quite characteristic of pregnancy, and patients who have previously been pregnant refer to the nausea as resembling that of pregnancy. Androgenic side effects may occur following the use of Neosteron, Nilevar or Norlutin (29, 51). The incidence of such effects appears to be highest with Neosteron and lowest with Norlutin. Enovid, when administered for long periods of time and even after high dosage in patients with endometriosis, has not been observed to produce androgenic effects (52, 53).

Methyl testosterone has been reported to produce jaundice (61) so that it is not surprising that its nor-derivative, Neosteron, has been observed to produce similar effects on the liver (51, 62). Jaundice (63) and BSP retention (64) have also been reported following 17-ethyl-19-nortestosterone (Nilevar). It appears that all of the alkyl derivatives of nortestosterone studied to date in man, including 17-ethynyl-19-nortestosterone (Norlutin) (65), increase BSP retention in many patients, and are potential producers of jaundice. Any new derivatives of this type must be carefully explored in this area. One 17-alkyl compound, norethynodrel (Enovid), which has the double bond in the 5(10) position,

has not produced changes in BSP retention and has not caused jaundice.

SUMMARY

By modification of steroid structure, various investigators have prepared compounds with high oral progestational activity. This is particularly true of derivatives of testosterone, nortestosterone, progesterone, and 17-OH-$\Delta^{5(10)}$ estrenolone. Certain derivatives may have practically only progestational effects, whereas other compounds may display varying degrees of anabolic, androgenic, pituitary inhibiting or anti-estrogenic properties. One compound, norethynodrel, has, for example, both progestational and estrogenic effects. Further, some of the newer progestins will maintain pregnancy in animals and others will not.

From the standpoint of fertility control, the subject of this symposium, norethynodrel, or Enovid, can effectively prevent fertility in the experimental animal without producing any untoward effects. The literature indicates a similar effectiveness and safety in normal women. The mechanism by which Enovid appears to act has been discussed.

REFERENCES

1. Inhoffen, H. H. and H. Hohlweg. *Naturwissenschaften* 26: 96, 1938.
2. Wilkins, L., H. W. Jones, Jr., G. H. Holman and R. S. Stempfel, Jr. *J. Clin. Endocrinol.* 18: 599, 1958.
3. Drill, V. A. and B. Riegel. In: *Recent Progress in Hormone Research*. New York; Acad. Press, XIV: 1958, p. 29.
4. Symposium. *Ann. New York Acad. Sci.* 71: 479, 1958.
5. Clauberg, C. *Zbl. Gynäk.* 54: 2757, 1930.
6. McGinty, D. A., L. P. Anderson and N. B. McCullough. *Endocrinology* 24: 829, 1939.
7. Lutwak-Mann, C. and C. E. Adams. *J. Endocrinol.* 15: 43, 1957.
8. Adams, C. E. and C. Lutwak-Mann. *Endocrinology* 13: 26, 1955.
9. Pincus, G., T. Miyake, A. Merrill and P. Longo. *Endocrinology* 63: 528, 1957.
10. Miyake, T. and G. Pincus. *Endocrinology* 63: 816, 1958.
11. Allen, M. and M. Ehrenstein. *Science* 100: 251, 1944.
12. Tullner, W. W. and F. Hertz. *Endocrinology* 52: 359, 1953.
13. Hertz, R., W. Tullner and E. Raffelt. *Endocrinology* 54: 288, 1954.
14. Hertz, R., J. H. Waite, and L. B. Thomas. *Proc. Soc. Exper. Biol. & Med.* 91: 418, 1956.
15. Tyler, E. *J. Clin. Endocrinol.* 15: 881, 1955.
16. Greenblatt, R. B. *J. Clin. Endocrinol.* 16: 869, 1956.
17. Drill, V. A. and F. J. Saunders. In: *Hormones and the Aging Process*, edited by E. T. Engle and G. Pincus. New York: Acad. Press, 1956, p. 99.
18. Pincus, G., M. C. Chang, M. X. Zarrow, E. S. E. Hafez, and A. Merrill. *Endocrinology* 59: 694, 1956.
19. Colton, F. B., L. N. Nysted, B. Riegel and A. L. Raymond. *J. Am. Chem. Soc.* 79: 1123, 1957.
20. Saunders, F. J., F. B. Colton, and V. A. Drill. *Proc. Soc. Exper. Biol. & Med.* 94: 717, 1957.
21. Overbeek, G. A. and J. deVisser. *Acta endocrinol.* 22: 318, 1956.
22. McGinty, D. A. and C. Djerassi. *Ann. New York Acad. Sc.* 71: 500, 1958.
23. Ferin, J. *Acta endocrinol.* 22: 303, 1956.
24. Hartley, A. D., D. R. Millison and V. Petrow. *J. Pharm. & Pharmacol.* 9: 939, 1957.
25. MaGrath, D., V. Petrow and R. Royer. *J. Chem. Soc.* 13: 845, 1951.
26. Greenbalgh, C. W., H. B. Henbest and E. R. H. Jones. *J. Chem. Soc.* 73: 1190, 1951.
27. Pincus, G., M. C. Chang, E. S. E. Hafez, M. X. Zarrow and A. Merrill. *Science* 124: 890, 1956.
28. Saunders, F. J., R. A. Edgren, and V. A. Drill. *Endocrinology* 60: 804, 1957.
29. Epstein, J. A., H. S. Kupperman and A. Cutler. *Ann. New York Acad. Sc.* 71: 560, 1958.
30. Southam, A. L. *Ann. New York Acad. Sc.* 71: 666, 1958.
31. Fried, J., W. B. Kessler and A. Borman. *Ann. New York Acad. Sc.* 71: 495, 1958.
32. Weid, G. L. and M. E. Davis. *Ann. New York Acad. Sc.* 71: 599, 1958.
33. Byrnes, W. W. and E. G. Shipley. *Endocrinology* 57: 5, 1955.
34. Perez Ruelas, J., J. Iriarte, F. Kincl and C. Djerassi. *J. Org. Chem.* 23: 1744, 1958.
35. Pfiffner, J. J. and H. B. North. *J. Biol. Chem.* 139: 855, 1941.
36. Junkmann, J. *Arch. exper. Path. u. Pharmakol.* 223: 244, 1954.
37. Kessler, W. B. and A. Borman. *Ann. New York Acad. Sc.* 71: 486, 1958.
38. Boschann, H. W. *Ann. New York Acad. Sc.* 71: 727, 1958.
39. Junkmann, K. and H. Witzel. *Ztschr. Vitamin-Hormon-u. Fermentforsch.* 9: 97, 1957–58.
40. Junkmann, K. and H. Witzel. *Ztschr. Vitamin-Hormon-u. Fermentforsch.* 9: 227, 1957–58.
41. Elton, R. L. In press.
42. Campbell, J. A., J. C. Babcock and J. A. Hogg. *J. Am. Chem. Soc.* 80: 4717, 1958.
43. Babcock, J. C., E. C. Gutsell, M. E. Herr, J. A. Hogg, J. C. Stucki, L. E. Barnes and W. E. Dulin. *J. Am. Chem. Soc.* 80: 2904, 1958.
44. Greenblatt, R. B. and W. E. Barfield. *South. M. J.* 52: 345, 1959.
45. Hogg, J. A., G. B. Spero, J. L. Thompson, B. J. Magerlein, W. P. Schenider, D. H. Peterson, O. K. Sebek, H. C. Murray, J.

C. Babcock, R. L. Pederson and J. A. Campbell. *Chem. and Ind.* (No. 32), p. 1002, 1958.
46. Bowers, O. and H. J. Ringold. *Tetrahedron* 3: 14, 1958.
47. Meystre, C. and A. Wettstein. *Helv. Chim. Acta* 32: 1978, 1949.
48. Saunders, F. J. and V. A. Drill. *Ann. New York Acad. Sc.* 71: 516, 1958.
49. Saunders, F. J. and R. L. Elton. In: *Endocrinology of Reproduction.* C. Lloyd, Ed., New York: Acad. Press, 1959, p. 227.
50. Smithberg, M. *Ann. New York Acad. Sc.* 71: 555, 1958.
51. Seelen, J. C. *J. Clin. Endocrinol.* 18: 1137, 1958.
52. Kistner, R. W. *Am. J. Obst. & Gynec.* 75: 264, 1958.
53. Kistner, R. W. *Postgrad. med. J.* 24: 505, 1958.
54. Edgren, R. A. *Endocrinology* 62: 689, 1958.
55. Ringold, H. *Ann. New York Acad. Sc.* 71: 514, 1958.
56. Rock, J., G. Pincus and C. R. Garcia. *Science* 124: 891, 1956.
57. Rock, J., C. R. Garcia and G. Pincus. In: *Recent Progress in Hormone Research.* New York: Acad. Press, 1957, XIII: 323.
58. Garcia, C. R., G. Pincus and J. Rock. *Am. J. Obst. & Gynec.* 75: 82, 1958.
59. Pincus, G. VI International Conference on Planned Parenthood, New Delhi, India, February 14–21, 1959.
60. Drill, V. A. VI International Conference on Planned Parenthood, New Delhi, India, February 14–21, 1959.
61. Kaplan, A. A. *Gastroenterology* 31: 384, 1956.
62. Peters, J. H., A. H. Randall, Jr., J. Mendeloff, R. Peace, J. C. Coberly and M. B. Hurley. *J. Clin. Endocrinol.* 18: 114, 1948.
63. Dunning, M. F. *J. A. M. A.* 167: 1242, 1958.
64. Kory, R. C., M. H. Bradley, R. N. Watson, R. Callahan and B. J. Peters. *Am. J. Med.* 26: 243, 1959.
65. Carbone, J. V. In press.

DISCUSSION

Daniel A. McGinty

Parke, Davis & Co., Detroit, Michigan

In opening the discussion of Dr. Drill's comprehensive review of the biological effects of progestational steroids, two experimental observations briefly mentioned by Dr. Drill, should be emphasized. First, progesterone itself and two other naturally occurring progestins of the corpus luteum and placenta, 20α- and 20β-Δ^4-pregnenolone, are far less active on oral administration than by injection. Secondly, by contrast, 17α-acetoxyprogesterone and several 17α-substituted derivatives of testosterone and especially of 19-nortestosterone are active by the oral route as well as by injection and in some instances are far more potent than progesterone. Introduction of the 6α-methyl group and presence of 9α-bromo or 9α-fluoro substituents enhances oral progestational activity. The comparatively low oral progestational activity of progesterone may be due in part to poor absorption from the intestinal tract and in part to rapid destruction by the liver. 17-Acetoxy progesterone and the 19-nortestosterones, in contrast to progesterone are rapidly absorbed after oral administration and apparently have a longer circulatory half-life than does progesterone.

The method employed for estimation of progestational activity requires some comment since it has become increasingly clear that comparative potencies of even chemically similar agents are dependent not only on the method of assay but on details of procedure as well. The Clauberg method and modifications of it is based on changes in endometrial histology of the estrogen-primed immature rabbit following a few days of treatment with the progestational compound. Pincus and associates (1) have recently enhanced the quantitative aspects of this test by planimetric measurements of the ratio of area of progestational proliferation to total mucosal area. Pincus and associates have demonstrated also that the observed increase in carbonic anhydrase content of the uterus of the Clauberg rabbit with increasing doses of injected progesterone or with certain orally administered progestational steroids correlates exceedingly well with estimates of activity as determined by glandular proliferation.

For progesterone, one can demonstrate an excellent dose-response pattern when the compound is injected directly into the lumen of the Clauberg rabbit (2) but one may or may not see this local progestational response with certain other steroids which are highly potent when injected subcutaneously or administered orally (3). Why certain progestogens fail to elicit a response when applied locally to the uterus of the Clauberg rabbit, is not clear. We would not agree with Dr. Drill's observation that only substances which exhibit a progestation reaction by the local test are able to maintain pregnancy in ovariectomized pregnant rabbits or rats. As we shall see,

maintenance of pregnancy in absence of the ovary, depends to a considerable extent on the mode of administration of the progestational agent.

The observation (4) that uteri of ovariectomized rats and mice treated first by estrogen priming and then with increasing doses of progestogens, will exhibit a correspondingly increased decidual growth when traumatized, has been utilized as an assay method. One horn of the uterus may serve as a control for the opposite horn. Another test for progestational activity which has been adapted for quantitative bioassay purposes (5), is based on the observation that increasing doses of progestogens will increase the frequency of implantation and total number of implanted ova in rabbits ovariectomized one day post-coitum. In this connection, it is well to point out that many of the progestational steroids, including progesterone itself will, with adequate dosage, prevent ovulation through inhibition of pituitary gonadotropin secretion.

Estrogen-stimulated growth of the oviduct in chicks was shown by Hertz and co-workers (6) and by others to be inhibited by progesterone and other progestational compounds. However, practical utility of the method for comparative studies has been questioned on the grounds of specificity of the antagonism by progestogens only.

Finally, tests for progestational activity which have come into increasing use are based on the observed prompt resorption or expulsion of fetuses of mice, rats or rabbits, ovariectomized early in gestation, and the maintenance of pregnancy with survival of living fetuses in such animals when treated adequately with progesterone or orally active progestogens.

In connection with one or another of such assay methods, I should like now, to call attention to wide differences in comparative potencies that have been reported for certain progestogens, in some instances by essentially the same test, in other instances by dissimiliar procedures when discrepancies in activity estimates may not be wholly unexpected.

Recently, Miyake and Pincus (7) compared 17α-methyl-, 17α-ethyl- and 17α-ethinyl-19-nortestosterone as well as 6α-methyl-17α-acetoxyprogesterone both orally and by injection using the Clauberg rabbit. Estimates of activity were based both on ratios of glandular proliferation to total mucosal area of uterine segments and on carbonic anhydrase content and were derived from dose-response curves and extent of maximal response. Both methyl-19-nortestosterone and ethinyl-19-nortestosterone were reported to be about one-tenth as active as progesterone when administered by subcutaneous injection whereas ethyl-19-nortestosterone was nearly 3 times as active as progesterone. These data are in contrast with those reported earlier from the same laboratory when it was found that methyl-, ethyl- and ethinyl-19-nortestosterone were all three equally active in the Clauberg test and in the order of 10 times as potent as progesterone. In the earlier tests however, potency estimates were made from minimal effective doses rather than from dose-response curves. These earlier data from the Worcester laboratory are in conformity with what we found in our laboratory, namely, that methyl-, ethyl- and ethinyl-19-nortestosterone all exhibited about equal progestational activity in the order of 5–10 times that of progesterone and furthermore that all three were about as active orally as by injection. Zarrow and co-workers also reported that ethyl- and ethinyl-19-nortestosterone was equally active in the Clauberg test with a potency about 10 times that of progesterone. The recent studies of Miyake and Pincus showed that ethyl-19-nortestosterone was 9 times as active as ethinyl-19-nortestosterone orally in the Clauberg test. 6αMethyl-17α-acetoxyprogesterone is a potent progestational compound. Miyake and Pincus found it to be 30–40 times as active as progesterone by injection in the Clauberg rabbit and 14–26 times as active as ethinyl-19-nortestosterone orally in the same test animal. Upjohn workers also reported it to be 25–100 times as active as progesterone by several tests.

In recent months, for estimation of progestational activity we, in our laboratory, have given increasing emphasis to a test depending for activity on maintenance of pregnancy in mice, ovariectomized at mid-gestation. The test has a number of advantages: *a*) it involves a most important function of progesterone itself, that of maintenance of the proper uterine environment for survival of living term fetuses, *b*) it allows for estimation of inadequate dosage or partial activity from the extent of fetal development, *c*) adaptability to various oral and injection treatments and *d*) ready availability of mice of dated pregnancy.

By this test, progesterone at a dose of 1 mg daily, subcutaneously in oil, will maintain pregnancy in 90% of treated mice. At a dose of 0.5 mg daily, approximately a third of treated mice will bear term fetuses.

Pregnancy cannot be maintained in the mouse with either methyl-19-nortestosterone or with the ethyl- or ethinyl- analogs with doses as high as 5 mg administered either subcutaneously in oil or orally in propylene glycol if treatment is given once daily. However, if either of these three steroids is mixed with the diet so as to provide a more or less continuous intake, all of the three compounds are active and quantitatively will

maintain pregnancy in the majority of treated mice at an intake of 2 mg daily and in about half of the mice at .6 mg daily. Furthermore, we have shown that while single daily doses of 2 mg ethinyl-19-nortestosterone, administered subcutaneously in oil, are wholly ineffective in the pregnant mouse, the same quantity of hormone administered once daily in oil as the hexahydrobenzoate or enanthate ester, permitted development of term fetuses in most of the treated mice. The obvious conclusion to be drawn from these experiments is that certain progestational steroids exhibit activity when administered in such a way as to provide a more or less continuous supply of hormone for the organism whether by constant feeding or by depot form of injection, whereas the same substance may appear to be inactive when administered intermittently in single daily doses. As mentioned earlier, once daily injections subcutaneously of .5 mg progesterone in oil will maintain pregnancy in about a third of treated ovariectomized mice whereas to achieve the same fetal survival rate at least 2 mg are required if injected every other day.

We found that 6α-methyl-17-acetoxy progesterone would maintain pregnancy in ovariectomized mice with single daily doses of 2 mg subcutaneously in oil but failed entirely at this same dose if given once daily orally. If, however, the compound was incorporated in the mouse diet so as to provide a daily intake of 1.9 mg, of 10 mice given this treatment, 8 had living term fetuses at laparotomy on the 19th day of pregnancy. In another experiment, in which the average daily intake of 6α-methyl-17-acetoxy progesterone was .6 mg from the diet, 4 of 10 treated mice had one or more term fetuses. Since the 50% fetal survival dose of 17α-ethinyl-19-nortestosterone was also .6 mg daily when the compound was mixed with the diet, it was concluded that progestational activity of these two compounds by this test was essentially the same.

These data are given as further evidence that relative effectiveness of progestogens is dependent not only on the particular test chosen for comparison but on the route of administration and frequency and duration of treatment.

REFERENCES

1. Pincus, G., T. Miyake, A. P. Merrill and P. Longo. *Endocrinology* 61: 528, 1957
2. McGinty, D. A., C. P. Anderson and N. B. McCullough. *Endocrinology* 24: 829, 1939.
3. Zarrow, M. X., G. M. Neher, E. A. Lazo-Wasem and H. A. Salhanick. *J. Clin. Endocrinol.* 17: 658, 1957.
4. Ashwood, E. B. *J. Endocrinol.* 1: 49, 1939.
5. Pincus, G. and N. T. Werthessen. *Am. J. Physiol.* 124: 484, 1938.
6. Hatz, R., C. D. Larsen and W. W. Tullner. *J. Nat. Cancer Inst.* 8: 123, 1947.
7. Miyake, T. and G. Pincus. *Endocrinology* 63: 816, 1958.

EFFECTS OF CERTAIN 19-NOR STEROIDS ON REPRODUCTIVE PROCESSES AND FERTILITY[1]

GREGORY PINCUS, JOHN ROCK, M. C. CHANG AND C. R. GARCIA

Worcester Foundation for Experimental Biology, Shrewsbury and the Free Hospital for Women, Brookline, Massachusetts

IN A SERIES of papers published from 1953 to date (1–7) we have presented data indicating that: *a*) progesterone and certain synthetic progestins, particularly the 19-nor steroid gestagens, are potent inhibitors of ovulation; *b*) certain of the 19-nor steroids have a high oral activity; *c*) these compounds exert characteristic progestational effects on the uterine endometrium; *d*) the sustainment of the uterine endometrium is facilitated when these progestins are administered along with a small amount of estrogen; *e*) a regular succession of anovulatory cycles of normal length may be had by the daily cyclic administration of a 19-nor steroid-estrogen combination over many months; and *f*) an oral contraceptive practice may thereby be achieved. In this paper we propose concentrating on the results of certain field studies of contraception in which the standard procedure has been the taking for 20 days, from the 5th through the 24th day of the menstrual cycle, of a 10 mg tablet of norethynodrel ordinarily containing 0.15 to 0.23 mg of the 3-methyl ether of ethinyl estradiol.

The details of the procedures employed have been published previously (7–9). It suffices here to state that each subject is given a vial containing 20 of the 10 mg tablets and told to take one tablet a day. Usually at about the time of the expected menstruation each subject is visited (or comes to a central clinic) and receives an additional month's supply. At the same time information is elicited concerning: *a*) the number of days (if any) when she forgot to take a tablet, *b*) the occurrence of any menstrual irregularities, *c*) the occurrence of any 'reactions' attributed to the medication, *d*) the frequency of sexual intercourse and *e*) any special problems that may have arisen.

[1] The work reported in this paper has been aided by research grants from G. D. Searle & Co., The Planned Parenthood Federation of America and Mrs. Stanley McCormick.

CONTRACEPTIVE PROJECTS

To November 1958, we have collected data on four projects: *a*) one initiated in April 1956 and located in San Juan, Puerto Rico; *b*) one initiated in April 1957 in a slum area in Humacao, Puerto Rico (designated Humacao-R); *c*) one initiated in June 1957 among a group of maternity clinic patients in Humacao (designated Humacao-P); and *d*) one initiated in December 1957 at Port-au-Prince, Haiti. The total number of subjects furnishing adequate information was 830, the number of active cases in November was 519, and the total number of cycles of tablet-taking was 8133, representing 635 woman-years of experience.

EFFECTS ON FERTILITY

Among the women taking medication, 17 pregnancies occurred, a rate of 2.7 per 100 woman-years, the lowest rate being 0.9 in the Humacao slum area and the highest 3.4 in Haiti. Among these women, all of proven fertility, the pregnancy rate previous to entering the project was 61.2 per 100 woman-years. This means that in 100 marriage-years 45.9 years were spent in the pregnant state and the balance, or 54.1 years, represent years of exposure to the risk of pregnancy. The crude rate per 100 exposure years is, therefore, 113. However, some allowance should be made for the sterile period normally following parturition as well as for the few cases in which some type of contraception was practiced. If we allow 3 months as the sterile post-partum period, then the rate per exposure years becomes 158, or allowing 6 months as the maximum (most of these women have suckled their children as long as possible) the rate becomes 273. Depending on which estimate one uses, the reduction in fertility varies from 97.6% to 99.4%.

If we classify the pregnancies according to the number of days of tablet-taking missed, we find that one conception occurred during a cycle in which no tablets were allegedly missed, giving a

rate of 0.2 per 100 woman-years; 5 pregnancies occurred in cycles in which 1–5 tablets were missed, to yield a rate of 7.7 per 100 woman-years; and 11 pregnancies occurred in cycles in which 6–19 tablet-takings were missed, giving a rate of 43.3 per 100 woman-years. It is clear that practically 100% effectiveness is had when faithful tablet-taking occurs, but even if tablet-missing occurs, significant contraceptive activity supervenes. The conception rate appears to be highest in the earlier medication cycles, e.g. 3.8 per 100 woman-years in the 3417 medication cycles 1–5 inclusive, and thereafter declines, indicating the reduction with experience of failures to follow the medication schedule. The very low conception rate in long-time medicated women certainly indicates no escape with time from the contraceptive effect.

Since by every criterion available, including direct inspection of the ovaries of users, ovulation inhibition uniformly occurs in each cycle of regular medication, the question has arisen as to the capability of the ovaries for ovulation following cessation of use. Clinical studies of subjects following 3–4 months' medication indicate prompt return of ovulation, ordinarily in the 1st menstrual cycle following cessation but always by the 2nd cycle (4). We have followed up 86 subjects in the San Juan project who withdrew from the project. Fifty-four of the 86 failed to use other contraceptive methods except rarely. Fifty-six percent (I) had taken the medication for 1–5 cycles, 18% (II) for 6–10 cycles and 26% (III) for 11–20 cycles. The overall conception rate was 197 per 100 exposure years; for the three groups the rates were: I—194, II—240, III—172, indicating no diminution of fertility with continued use at least for the periods of medication involved. Incidentally, among the 32 who allegedly used other contraceptives regularly, there were 5 pregnancies with a rate of 27 per 100 exposure years.

EFFECTS ON MENSTRUAL PHENOMENA

In the normal course of a medication cycle it is to be expected that following the taking of the last tablet a withdrawal bleeding will occur, since sustainment of the endometrium is no longer afforded. Actually in the great majority of cases studied this withdrawal menstruation occurs after 1–5 days (4, 5). In a number of instances, however, bleeding may occur during the course of medication. This is termed 'breakthrough bleeding' and represents inadequate endometrial sustainment. In the subjects of this study the time of menstruation was regularly reported, and it thus appears possible to estimate the frequency of occurrence of inadequate endometrial sustainment. Since, however, there were occasional misses in the taking of tablets, bleeding during medication may represent not breakthrough but a withdrawal effect. Examination of our data discloses that no tablet-missing was reported in 6931 medication cycles and that cycles of less than 24 days[2] in length occurred in 1.8% of these 6931 cycles; in the 871 cycles wherein 1–5 tablet-takings were missed 23.5% of the cycles were less than 24 days in length; and in the 331 cycles wherein 6–19 tablet-takings were missed, this percentage is 25.7. It would appear, therefore, that failure to follow the medication regime faithfully will result in premature menstruation in up to 26% of the cycles, and that this premature menstruation is a withdrawal bleeding in most instances, since among the allegedly faithful users only 1.8% of short cycles occurred. Even this figure of 1.8% may be somewhat suspect since inaccurate reporting may be involved, but to an unknown extent. It is interesting to note that Haman (10) reporting on menstrual cycle lengths in 2410 cycles of 150 normal American wives found that cycles of 23 days or less occurred in 5.2% of the total cycles. This clearly exceeds the 1.8% observed in the faithful tablet-takers, and, indeed, indicates that when regularly employed the medication regime will regularize menstrual frequency. The coincidence that the overall incidence of short cycles among the 8133 recorded is 5.1% suggests that nature makes approximately the same mistakes in furnishing endometrial sustainment as do the tablet-takers of our study.

An examination of our records on premature menstruation discloses that in each project the highest frequency of occurrence is in the first cycle of medication. These frequencies for San Juan, Humacao-R, Humacao-P and Haiti are 4.8%, 6.0%, 11.9% and 6.7%. There is thereafter a decline to rather constant low levels, varying from 0.0% to 3.3% for any given set of medication cycles. This high frequency in the 1st medication cycle may be due, at least in part, to inexperience and forgetfulness; it is also possible

[2] Cycles of less than 24 days are considered premature since the tablet taken on the 24th day might conceivably be endometrium-sustaining for only several hours.

that the use of exogenous 'hormone' involves an endometrial readjustment from its response to endogenous hormone to the special early progestational stimulation of the medication. In the subsequent cycles the endometrium is perhaps 'prepared' for the early progestational effect.

Occasionally withdrawal of the medication fails to be followed by menstruation. Such amenorrhea has been observed in 0.7% of the recorded menstrual cycles in these studied. If, despite the absence of menstruation, the subject initiates a renewed course of medication within 10 days after cessation, this renewed course will be followed by the usual withdrawal bleeding. If, however, the subject is pregnant, withdrawal bleeding will not occur, and in two instances failure of withdrawal bleeding has occurred in non-pregnant subjects after this second course.

We have inquired of samples of our subjects concerning the degree of menstrual flow. Increased flow was reported by 6%, no change by 46% and a decrease by 48%. There was some indication of the alleviation of dysmenorrhea in medication cycles 1–19 (among 77 subjects), but in 59 subjects in cycles 20–37, accentuation and alleviation scored equally. However, approximately 75% of all subjects questioned reported no change, so that significant assessment of effect is dubious with the numbers involved.

EFFECTS OF LIBIDO, SEXUAL BEHAVIOR, AND WELL-BEING

The subjects questioned about menstrual flow and pain were also questioned about possible changes in sexual libido. Among the 78 responding, 13 reported an increase, 20 a decrease and 45 no change; these reported changes are not statistically significant, nor was there any consistent relationship to the number of cycles of medication. Frequency of coitus was regularly reported for pre-medication months and from the first medication cycle on by the subjects of the Humacao-R, Humacao-P and Haiti projects; on the average there was a mean increase in each group in monthly frequency through the 8th medication cycle and a leveling off thereafter. Actually 50%–52% of the subjects reported increased frequencies, 34%–42% decreased frequencies and 6%–16% no change. In the San Juan project a group of 31 women reporting for the 5th to 32nd cycles of medication had an unchanged average of approximately 10 per month throughout, and a group of 73 reporting for the 1st to 20th medication cycles had similarly a constant frequency of 9.8 per month.

Among 57 patients responding to questioning concerning general health and well-being, 30 reported no change (53%), 21 an increase (37%) and 6 a decrease (11%). Of the 35 taking medication for 20 or more cycles, 60% reported no change, 34% an increase and 6% a decrease. Of 73 responding to questioning about weight changes, 57% reported weight increases, 23% no change and 20% weight decreases on medication. It is clear from these data that adverse effects on well-being are minor and that an anabolic effect of the medication may occur. We are inclined to believe that the relief from anxiety concerning possible pregnancy may be responsible for the situation indicated, i.e. less anxiety leading to less tension and more eating.

SIDE EFFECTS

A proportion of the subjects taking this medication have reported undesired side effects such as dizziness, nausea, vomiting, gastralgia, and lower abdominal pain. Such symptoms were reported in 10.9% of 4988 medication cycles in San Juan, in 6.3% of 1410 cycles in Humacao-R, in 18.3% of 658 cycles in Humacao-P and in 7.3% of 1077 cycles in Haiti. Actually, the highest frequency of occurrence of such reactions was in each group in the first medication cycle, the percentages of occurrence in this cycle being 16.9, 29.9, 38.1 and 22.1, respectively. Thereafter the frequency declines markedly to levels characteristic for each group usually by the 4th medication cycle, and is respectively for each of the 4 groups approximately 10% (up to 37 cycles), 2% (up to 20 cycles), 9% (up to 18 cycles) and 4% (up to 11 cycles). The high frequencies reported for 1st cycles suggest again a uterine accommodation to the medication, perhaps in the nature of the myometrial response.

Some of the symptoms may reflect an effect on the liver, and accordingly we conducted 2 liver function tests (thymol turbidity and cephalin flocculation) with 20 control subjects and 148 taking the medication. The average results in both tests did not significantly differ in the control and experimental subjects. One subject on medication had values outside the normal range for these tests, an occurrence to be expected in West Indian women of this age range.

Since no evidence of deranged liver function could account for the symptoms, a psychogenic

origin should be suspected. The high 1st cycle frequencies suggest this, since apprehension concerning the use of a novel type of contraception might be maximal at that time. We have attempted to test the possibility of a psychogenic basis by studying two groups of women in Puerto Rico. Group (a), consisting of 28 women regularly using other types of contraceptives who were given 'pills' to be taken for a few 'preparatory' cycles without abandonment of their regular contraceptive practice, were studied in a double-blind experiment in which a random distribution of pills denominated (to the distributor but not to the subject) tablets A and B was made. Group (b), consisting of 15 women in a town 15 miles from the area occupied by the women of Group (a), were offered 'contraceptive pills' with no admonition concerning possible side effects. Thirteen women of Group (a) received true medication (tablet A) and were visited for 30 medication cycles; fifteen women of Group (a) received placebos (tablet B) and were visited for 41 medication cycles; comparable data on the 15 women of Group (b) were obtained for 48 medication cycles.

No significant difference was found in the frequency of reported reactions in the placebo and true medication subjects of Group (a); reactions were reported in 17.1 ± 5.9% and 23.3 ± 7.7% of the medication cycles respectively. The women of Group (b) reported reactions in 6.3 ± 3.5% of the medication cycles. A psychogenic factor is clearly indicated. It is interesting to note that the percentages of breakthrough bleeding in the A, B, and no admonition groups were 16.7, 4.9 and 2.1, respectively, and the percentages of amenorrhea were 3.3, 9.8 and 0.0. Psychological influences on menstrual phenomena are suggested.

ACCEPTABILITY

We may examine the degree of acceptability of this type of contraception by two sets of data. The first is concerned with the faithfulness of tablet-taking. Among the 8133 cycles of medication, no missing of tablet-taking was reported in 6931 or 85%, the missing of 1–5 tablets was reported in 871 or 11%, and of 6–19 tablets in 331 or 4%. That the no misses probably represent true reporting is indicated by the very low frequency of premature bleeding in this group (*vide supra*). This record of faithful tablet-taking in rather busy women of low economic status seems to us to be a good one.

The second set of data is concerned with the rates of withdrawal from the projects. By the end of the first year, 50% of the women starting in the San Juan project had withdrawn; among those continuing, 30% withdrew by the end of the second year. Thus, after two years only 35% of the starters remained. On examining the reasons for withdrawal, we find that 15% had moved away (chiefly to the continental United States), 13% had been sterilized, 9% had been separated from their husbands, 7% left because of unrelated illness, 5% left because they were pregnant and 7% left for miscellaneous reasons (including husbands' opposition, 2%; prostitution, 1%; religion, 1%). Of the remainder, 28% left because of alleged 'reactions' to the medication and 16% because of lack of interest, i.e. poor motivation.

The highest withdrawal rates occurred in the San Juan project. In the other projects the rates of leaving at the end of the first year were 11%, 46%, and 34% for Humacao-R, Humacao-P and Haiti, respectively. The reasons for the differences between the various projects pertain to various factors such as mobility of the population, the availability of sterilization and other contraceptive measures, motivation, and so on. Detailed study from the psychological and sociological point of view should be interesting and rewarding. If we examine the data on reasons for leaving, it is clear that the risk of reproduction in any given community is incurred by approximately half of those leaving.[3] However, we have found by follow-up in San Juan that of these, approximately 30% undertake the regular use of other forms of contraception. It is obvious that alternatives to this particular mode of contraception are desired and desirable. Among potential forms of such alternatives are other types of oral contraception, and in a few years we hope to report on such alternatives.

REFERENCES

1. Pincus, G. and M. C. Chang. *Acta physiol. latinoam.* 3: 177, 1953.
2. Pincus, G. Proceedings V International Conference on Planned Parenthood, Tokyo, 1955, p. 175.
3. Pincus, G., M. C. Chang, M. X. Zarrow, E. S. E. Hafez and A. Merrill. *Endocrinology* 59: 695, 1956.

[3] Not by those sterilized, moving out of the community, separated from husbands by death or divorce, with disabling disease, etc.

4. Rock, J., C. R. Garcia and G. Pincus. *Recent Progr. Hormone Res.* 13: 323, 1957.
5. Pincus, G. Proc. Symposium on 19-nor Progestational Steroids. Searle Research Laboratories, Chicago, 1957, p. 105.
6. Pincus, G., J. Rock and C. R. Garcia. *Ann. N. Y. Acad. Sc.* 71: 677, 1958.
7. Pincus, G., J. Rock, C. R. Garcia, E. Rice-Wray, M. Paniagua and I. Rodriguez. *Am. J. Obst. & Gynec.* 75: 1333, 1958.
8. Pincus, G. *Stud. Fertil.* 10: 3, 1959.
9. Rice-Wray, E. Proc. Symposium on 19-nor Progestational Steroids. Searle Research Laboratories, Chicago, 1957, p. 78.
10. Haman, J. O. *Am. J. Obst. & Gynec.* 43: 870, 1942.

DISCUSSION

Robert B. Greenblatt

Medical College of Georgia, Augusta, Georgia

Dr. Pincus and his group are to be highly commended for a well-planned and well-executed project. This comprehensive study may serve as a model for clinical investigators to follow. In evaluating the effectiveness of 19-norsteroids as contraceptive agents, no amount of animal studies can take the place of clinical investigations. Dr. Pincus has furnished us with impressive figures showing that the 19-norsteroids have proved most effective in preventing conception. The goal of the study was to prevent conception, and many factors are at play when estrogens and progesterone are administered; there are ovulatory factors, the factor of ovum and sperm migration, both histologic and enzymatic endometrial changes, as well as the role of cervical mucus acting as a barrier to sperm. I should like to ask Dr. Pincus if he believes that the good results obtained with as little as a daily dose of 2.5 mg of norethynodrel and 0.0375 mg of 3-methyl ether of ethinyl estradiol, were due to inhibition of ovulation? It is doubtful whether such small doses effectively inhibit ovulation; nor am I entirely convinced that the standard dose of 10 mg of norethynodrel and 0.15 mg of 3-methyl ether of ethinyl estradiol per day, accomplished this with any degree of frequency. The success of this enterprise depended probably more on the induced endometrial changes that may well have proved inimical to implantation. The role of cervical mucus as a barrier to sperm has not been emphasized sufficiently and it is my considered opinion that the friendly clear watery mucus (capable of ferning on drying) which is a haven for the sperm in their struggle for survival, is changed to a hostile, thick, tenacious mucoid secretion (with loss of the fern phenomenon on drying). Should ovulation occur, in spite of the estrogen-progesterone medication, then the mucus changes are sufficient to prevent conception. Figure 1 shows the small amount of 6-methyl 17 acetoxyprogesterone needed to inhibit ferning of cervical mucus.

The dosages employed by Dr. Pincus and his group have been gradually lowered for several reasons—*a*) to lower the incidence of side effects, and *b*) to lower the cost of medication. It appears that though the incidence of side effects such as nausea were not substantially lowered in his series, nevertheless in our own experience such complications are far less frequently encountered when other progestational agents were employed. Furthermore, when the dosage schedule is greatly reduced, the costs are indeed lowered, but I fear that this occurs at the expense of breakthrough bleeding. To my way of thinking the increased incidence of breakthrough bleeding would serve to hasten withdrawal from the project.

Another of the side effects mentioned was a lessening of libido. Frequency of coitus was not appreciably reduced since the male was not

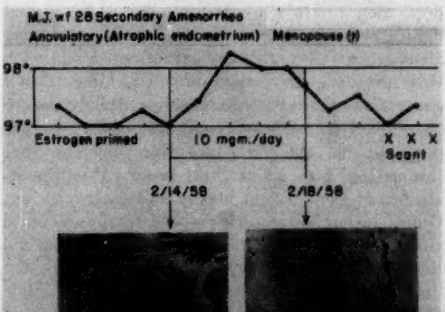


Fig. 1. The administration of 6 methyl 17-acetoxyprogesterone in 10 mg doses per day inhibited the phenomenon of cervical ferning within 4–5 days.

affected by the medication. Libido in the female is not a positive force but is best expressed in 'passive acquiescence'. In those females in whom libido is marked, prolonged progesterone therapy does appear to dampen their ardor.

Interesting indeed are Dr. Pincus' studies suggesting that the 19-norsteroids may modify adrenal corticosteroid excretion. Several years ago we were able to show that 17-hydroxyprogesterone caproate caused a marked lowering in excretion of urinary pregnanetriol in congenital adrenal hyperplasia without alteration in urinary 17-ketosteroids or 17-hydroxycorticoids.

The question of course will be asked—"How safe is the prolonged use of these progestational agents?" Dr. Pincus, I feel, has answered this question adequately by pointing out that many of the women who dropped out of the project conceived quite readily in spite of the fact that they were the recipients of months to years of 19-norsteroids. That progestational substances are safe and do not cause any lasting derangement in pituitary-ovarian balance is best shown by the fact that we have delayed menstruation for periods up to nine months in the treatment of endometriosis and resumption of ovulatory menses occurred soon after cessation of therapy.

In closing I again wish to compliment Dr. Pincus for a most enlightening presentation. I feel that he and his associates are on the right track and it is hoped that the ideal oral preparation will be found—with little breakthrough bleeding, minimal untoward reactions, and which will prove to be safe and effective in controlling conception.

REPLY TO DISCUSSION

GREGORY PINCUS

THAT THE 19-NOR STEROIDS in the standard 10 mg dose which we have used inhibit ovulation in women appears to be indubitable, both on the basis of the clear reduction in pregnanediol excretion and the results of direct observation of ovaries of women taking this dosage. These observations at laparotomy, originally made by Drs. Rock and Garcia, have been confirmed by an even larger series in Japan. Whether the 2.5 mg dose also inhibits ovulation cannot be stated definitively on the basis of the available evidence, although a few clinical observations made with this low dose are suggestive. Dr. Greenblatt's suggestions that either the endometrial changes or the effects upon the cervical mucus may be important in the contraceptive action, are interesting but await full proof. Preliminary studies of the cervical mucus undertaken by Dr. Guard in our laboratories would tend to confirm the notion that penetrability by sperm is indeed limited. However, very extensive data are required before this may be stated as a generality. Concerning the use of lowered dosages, I think that Dr. Greenblatt's comments are very appropriate, but we are now in the process of collecting data on the direct effects on the uterus, which should answer some of the questions he raises. One experiment, involving an alteration of the ratio of norethynodrel to estrogen, should be very instructive as to the possibility of the control of breakthrough bleeding.

In presenting data on field trials with one preparation, I did not mean to ignore the fact that a number of others may be practical as contraceptive agents, including the 6-methyl-17-acetoxyprogesterone mentioned by Dr. Greenblatt. I should say that there are now available a number of interesting progestins, and we plan to extend our studies to these in the future. However, we feel that every compound to be tested should have a searching clinical study before its employment in field trials. For example, in animal tests, 6-methyl-17-acetoxyprogesterone has proven to be a powerful inhibitor of adrenal function. If this is true also for women, both the qualitative and quantitative effects must be scrutinized in detail before it can be tested on a large scale for contraception.

EFFECTS OF PROGESTERONE AND SYNTHETIC PROGESTINS ON THE REPRODUCTIVE PHYSIOLOGY OF NORMAL MEN[1, 2]

CARL G. HELLER, DONALD J. MOORE, C. ALVIN PAULSEN, WARREN O. NELSON AND WILLIAM M. LAIDLAW

Pacific Northwest Research Foundation, Seattle, Washington, The Rockefeller Institute, New York City and Department of Medicine, University of Oregon Medical School, Portland, Oregon

A PAUCITY of information concerning the effect of steroids on the testicular-pituitary axis of normal men exists. Still less is known of the effects of progesterone, or the newer synthetic progestins. This is in large part due to a lack of subjects willing to have repeated observations made upon seminal fluid, urine and testicular biopsy specimens, let alone to take oral or intramuscular medication daily.

This difficulty was obviated by calling for volunteers at the Oregon State Penitentiary. The advantages in working with such volunteers are that they are maintained under uniform circumstances regarding diet, rest, overindulgences and work. They are available for observation at most times. Long-term studies (in this case, 2 years) may be undertaken with a minimal attrition rate and as we found, the cooperation of the subjects was excellent. A further advantage is that a number of steroids may be tested concurrently; for example, the first 20 subjects under discussion were started on medication simultaneously.

For this first contingent of volunteers we chose to compare the physiologic effects of 3 commercially-marketed progestins with progesterone itself.

Nilevar (17-α-ethyl-19-nortestosterone),[3] the first of these synthetic progestins, is known to inhibit ovulation and to produce a progestational-like endometrium in women (1). Its commonest current clinical usage, however, is based on its anabolic effect. Nilevar has found favor because of the dichotomy between its androgenicity and its anabolism (2, 3) and because it is highly effective when administered orally.

In order to determine its effects upon spermatogenesis in normal men, seven subjects were given 30 mg per day orally (20 mg each morning and 10 mg each evening).

Results. By the end of 4 weeks a perceptible decrease in sperm output and a marked decrease in sperm motility was noted. By the end of 8 weeks each of the seven men had azoospermia, complete loss of libido, a marked reduction in potentia and an alarming decrease in testicular size. The drug was discontinued at this juncture in four subjects, after 21 weeks in one and after 25 weeks in the remaining two. Upon discontinuing medication a second testicular biopsy and further urine specimens were obtained.

Effects upon testicular morphology were determined by studying bilateral testicular biopsy specimens before administering Nilevar, and unilateral biopsy specimens obtained at the time the drug was discontinued (see figs. 2–5). In each instance the same type and degree of alteration was observed, namely: *a*) a marked decrease in size of the seminiferous tubules, accounting for the gross decrease in testicular size; *b*) an apparent increase in the density of the peritubular membranes, occurring without concurrent hyalinization, as is seen after administering testosterone propionate or estrogen (this increase in density may be due to shrinkage in tubular size); *c*) hypoplasia of all germinal elements, including absence or marked reduction of spermatozoa, spermatids and secondary spermatocytes, and a great decrease in primary spermatocytes, with a moderate decrease in numbers of spermatogonia; *d*) the sustentacular cells of Sertoli remained in-

[1] Supported by grants-in-aid C-2673, CY-4240, RG-4823 and RG-5961 from the National Institutes of Health, Public Health Service, Bethesda, Maryland; The Population Council of The Rockefeller Foundation, New York 21, New York; and G. D. Searle & Co., Chicago 80, Illinois.

[2] This research program was made possible by the generous cooperation of Warden Clarence T. Gladden and Dr. Robert T. Boals of the Oregon State Penitentiary, Salem, Oregon.

[3] Kindly provided by Dr. Robert Craig of G. D. Searle & Company, Chicago.

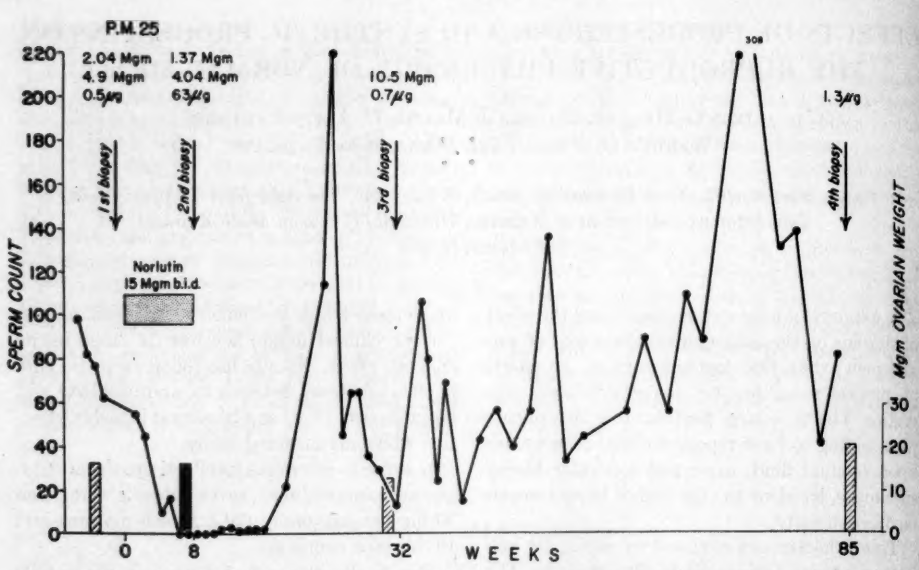


FIG. 1. Depicts effect of 8 week course of Norlutin on pituitary-gonadal function of PM-25. The connected dots denote spermatozoa concentration in millions per ml. The bars along the abscissa indicate the urinary gonadotrophin titer in mg ovarian weight. The shading denotes the presence of stimulated uteri (above 60 mg), whereas the solid bar denotes absent uterine stimulation. Thus urinary gonadotrophins were completely suppressed at the end of Norlutin administration. (16-hour urine aliquots were used in assaying this subject's urinary gonadotrophins.) At the top of the chart the values for urinary pregnanediol, 17-ketosteroid and estrogen excretion are given. Note no essential change in pregnanediol or 17-ketosteroid excretion. Urinary estrogens increased markedly from normal pretreatment levels.

tact; and finally *e*) disappearance of Leydig-cells, although interstitial mesenchymal cells were identifiable.

These histological changes occurred in six of the seven subjects. The seventh subject, who responded in consonance with the other subjects in each of the other parameters tested, unaccountably exhibited no change in testicular morphology.

Mechanism. Two questions arise: *1*) were these histological alterations wrought by interference with gonadotrophin secretion? Possibly, since gonadotrophins were detectable in the pretreatment urine samples in each of four subjects in which satisfactory observations were made, and were not detectable in any of the seven subjects at the end of treatment. *2*) Was the reduction in gonadotrophins due to an increase in estrogen? No, since pretreatment and end-of-treatment urinary estrogen assays for each of the seven were similar; in fact, there was a suggestion of estrogen suppression in several subjects.

Pregnanediol excretion was unaltered. 17-ketosteroid excretion was reduced slightly (from 8.4 ± 0.98 to 5.1 ± 0.55 mg per 24 hours and Standard Error) for the group. The likely explanation is that Nilevar is not converted to a 17-ketosteroid, and that the reduction in 17-ketosteroids is accounted for by the reduction in Leydig-cell function.

The androgenicity of Nilevar was insufficient to prevent the androgen withdrawal symptoms of the male climacteric as Leydig-cell function declined. In contrast, when testosterone is administered to normal men, it is sufficiently androgenic to substitute for the declining Leydig-cell function, and no climacteric symptoms appear.

The following anabolic effects of Nilevar were noted: increase in appetite, weight gain and increase in muscle mass and strength. In contrast, five men on the same dietary regimen who were receiving progesterone had no such effects.

Recovery phase. Libido and potentia reappeared in 2–3 weeks. The seminal fluid remained essentially azoospermic until the 12th posttreatment when the first significant rise in sperm counts occurred in five subjects, and at 9 and 13 weeks in the remaining two.

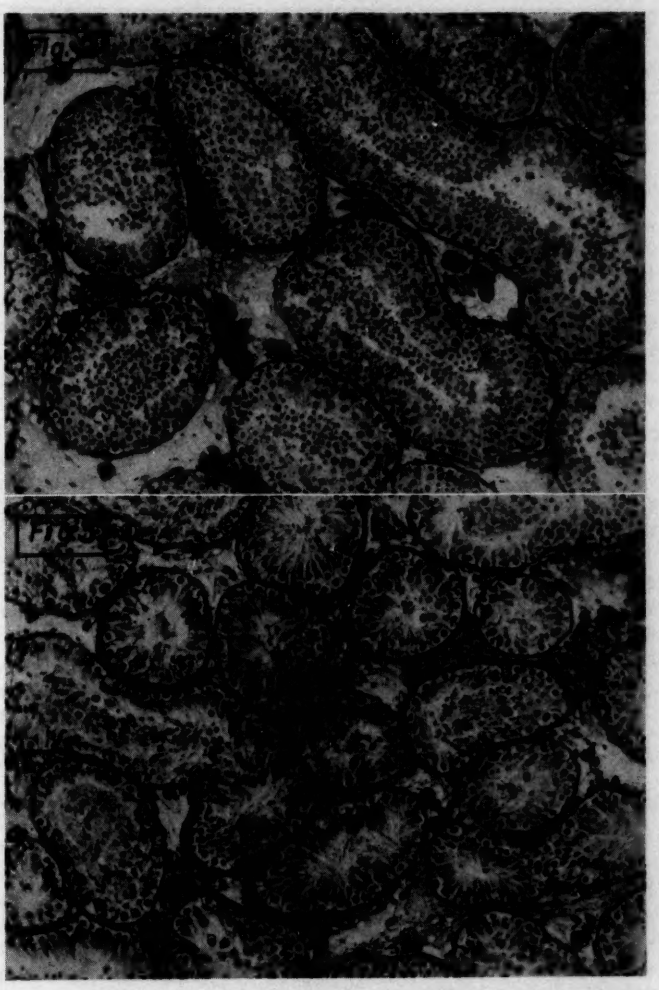


FIG. 2. Testicular biopsy specimen of subject PM-16 prior to 8 week course of Nilevar. Spermatozoa concentration of seminal fluid ranged from 50–76 million per ml. ×125.

FIG. 3. PM-16, after 8 weeks of Nilevar 15 mg twice daily orally. Note marked hypoplasia of the germinal epithelium and diminished tubular size. Reduction is mainly of mature forms, however occasional spermatids and mature sperm forms can be seen. Seminal fluid at this time revealed azoospermia. Minimal fibrosis of tubular membranes and vacuolization along with significant decrease in numbers of Leydig-cells were also noted. × 125.

Twenty-four to twenty-six weeks after discontinuing Nilevar, sperm counts reached a steady state at a level of sperm production distinctly above pretreatment levels in three subjects, the same as control levels in two and lower in two. Of the five who had biopsy specimens taken at this time, each revealed a distinct improvement over the previous hypoplastic state and Leydig-cells had reappeared. However, the seminiferous tubules exhibited great disorganization, and there was a distinct increase in germinal elements of all types, including many abnormal forms. Of considerable import is the appearance of basement membrane hyalinization during this recovery phase which is probably related to the *rapid* recrudescence of endogenous testicular hormone production. Urinary excretion studies revealed normal levels of gonadotrophins, 17-ketosteroids and estrogens.

The changes brought about by administering Nilevar may be interpreted as being a return toward the prepuberal state rather than actual damage. Thus the hormonal levels are reduced, Leydig-cells return to their mesenchymal state, seminiferous tubules are reduced in size and for the most part the chief germinal elements are spermatogonia.

Upon withdrawal of medication a recapitulation of the puberal process occurs, albeit quite rapidly. This includes a rapid rise in hormonal

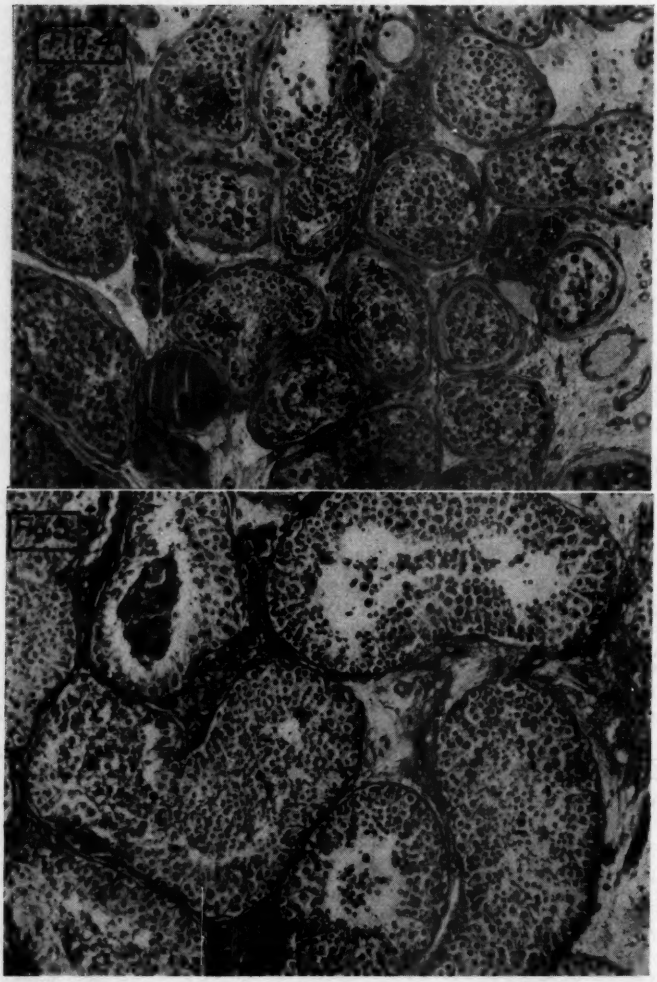


FIG. 4. PM-16, 25 weeks after cessation of therapy. Disorganization of spermatogenesis is the main feature. A moderate increase in germinal epithelium has occurred, but tubular size is still diminished. Note hyalinization has occurred. Leydig-cells now appear normal. Sperm counts at this time ranged from 25–76 million per ml. ×125.

FIG. 5. PM-16 final histologic observation at 70 weeks posttreatment. Spermatogenesis is essentially normal. Sperm counts 40–110 million per ml. ×125.

levels (4) followed by a rise in sperm count, while a lag in orderly arrangement of germinal elements is present. Even the occurrence of placque-like gynecomastia, so frequently observed during puberty, was noted evanescently in some subjects.

Final observations were completed on four subjects who received Nilevar for only 8 weeks, 70–82 weeks after discontinuing medication. Sperm output equalled pretreatment levels in two and rose above them in two.

Bilateral testicular biopsy specimens were obtained for all final posttreatment studies in this Nilevar group as well as in each of the subsequent groups. Histological testicular morphology was compared with the control testes, and complete recovery had taken place in each except that obtained from the left testis of PM-2 where disorganization of the germinal epithelium was still present.

It should be noted that the three subjects on long-term medication, i.e. 21 and 25 weeks, recovered as rapidly and as completely as the four in whom medication was discontinued as soon as azoospermia was reached. The suggestion had been made (by W.O.N.) that breakthrough might occur with prolonged therapy at the same dose level; however once azoospermia was attained, spermatozoa did not appear in the seminal fluid during therapy.

Norlutin (17 - alpha - ethynyl - 19 - nortestosterone)[4], is a progestin that induces progesterone-like endometrium and is alleged to inhibit ovulation (1). Although somewhat anabolic, its main clinical use at present is to regulate abnormalities of the menstrual cycle. As marketed it contains ethynylestradiol 3-methyl ether in addition to the parent substance. The preparation we used contained less than 0.2% of this contaminant, whereas the currently marketed preparation contains more.

Again, we chose five apparently normal men from the prison population. Each ingested 30 mg per day in divided doses.

Spermatogenesis was depressed to zero in 6 weeks (see fig. 1). At this time the subjects also had loss of libido and potentia, reduction in testicular size and two had developed gynecomastia. Drug administration was discontinued at the end of eight weeks in all subjects. Urine for hormonal assays was collected four days prior to a testicular biopsy specimen obtained at this time.

Effects upon testicular morphology were identical to that seen with Nilevar administration, i.e. uniformly small size of the seminiferous tubules without significant hyalinization, hypoplasia to the point where only spermatogonia could be discovered in three subjects, and only occasional other cell types in the remaining two. Leydig-cells were absent.

Mechanism. Was the alteration in testicular morphology due to depression in gonadotrophins? Possibly, since four of the subjects who had detectable gonadotrophin levels before treatment had none at the end of medication. In the fifth subject no gonadotrophins were detected in 12-hour aliquots assayed before treatment (and also none in 16-hour aliquots at the end of therapy). Could the gonadotrophin decrease be due to estrogen? Yes, since estrogens increased in each subject, and in two, where the supply of extract permitted reassays, urinary estrogen excretion increased from pretreatment levels of 0.5 and 0.6 μg-equivalents to 63 and 59.3 μg-equivalents of estradiol benzoate per 24 hours respectively. Thus the estrogen alone could account for the suppression of urinary gonadotrophins.

Pregnanediol excretion remained unaltered. 17-ketosteroid levels were reduced (from 6.4 ± 2.3 to 2.9 ± 0.8 mg per 24 hours and Standard Error) for the group.

This suggests that Norlutin is not metabolized in the same manner as progesterone, i.e. to pregnanediol, nor in the same manner as testosterone, i.e. to 17-ketosteroids. The reduction of the latter may be ascribed to the induced Leydig-cell failure.

Recovery Phase. In 2–3 weeks libido and potentia had returned and the first reappearance of sperm in significant numbers occurred in 10–11 weeks. A 3rd biopsy was obtained in four subjects when sperm production reached a steady state (at control levels for three and higher for one) 24–26 weeks after discontinuing Norlutin. The fifth subject was unavailable at this time because he had been released on parole (observations were continued upon his prompt return!). The hypoplastic nature of the testes had disappeared, but normal arrangement of germinal cells had not yet occurred. Urinary hormonal levels had returned to normal.

Final observations were made on each of the five subjects at 75, 77, 81, 82, and 87 weeks after discontinuing Norlutin. At this time sperm counts were higher than before medication in three, the same in one, and not obtained in one. Estrogen and gonadotrophin levels continued to be normal. Testicular morphology had recovered completely in three, improved in one, and one subject still exhibited disorganization and sloughing of germinal elements as well as some hypoplasia.

Enovid (17 - α - ethynyl - 17 - hydroxy - Δ - 5,10-estrene-3-one)[5], also causes progestational-like endometrial changes, inhibits ovulation and is anabolic. (1, 3) Its main clinical application is to regulate menstrual disorders. It, too, contains ethynylestradiol 3-methyl ether. The preparation we used, and the preparation marketed at present, contains 1.5% of the estrogen.

Enovid was administered orally to five normal subjects at the rate of 30 mg daily in divided doses. Spermatogenesis was suppressed to zero, as reflected by azoospermia of the seminal fluid, within 4½ weeks in three subjects and within 6 weeks in two others. This is a more rapid suppression than that occurring with Nilevar (8 weeks) or Norlutin (6 weeks). A concomitant decrease in libido, potentia and testicular size was observed, and gynecomastia developed in

[4] Lot No. X-6943, kindly provided by Dr. J. E. Gajewski, of Parke-Davis and Company, Detroit.

[5] Lot No. 927685, kindly provided by Dr. J. William Crosson, G. D. Searle & Company, Chicago.

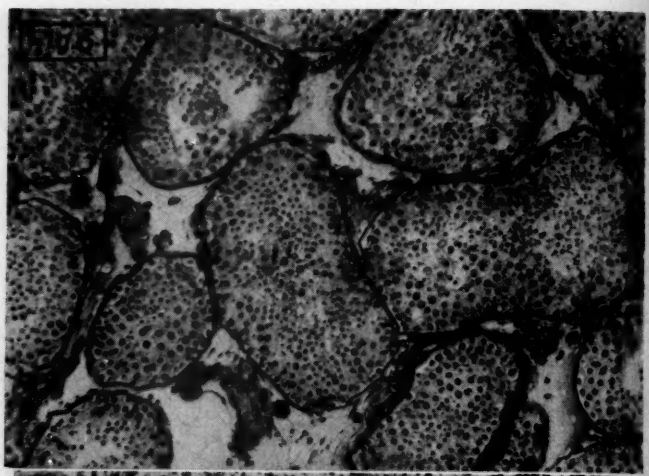


FIG. 6. Testicular biopsy specimen of subject PM-10 prior to 7 week course of Enovid. Spermatozoa concentration of seminal fluid ranged from 44–65 million per ml. ×125.

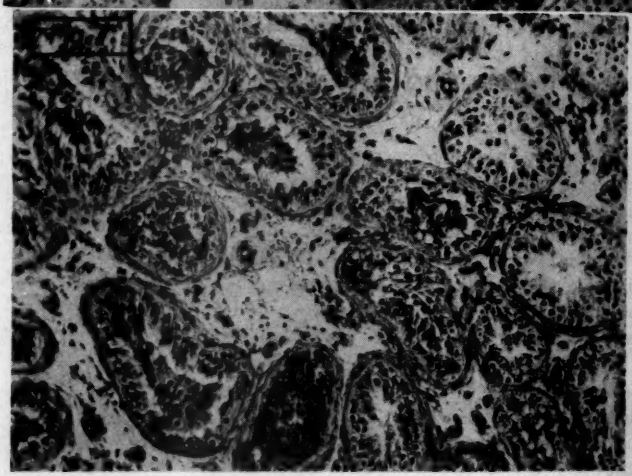


FIG. 7. PM-10 after 7 weeks of Enovid 15 mg twice daily. In contrast to the Nilevar treated subjects, minimal hyalinization of the basement membrane occurred. Note sloughing of the germinal epithelium and hypoplasia. ×125.

four of the five subjects. Medication was discontinued at the end of the 7th week. Four-day urine collections were obtained and biopsies were performed at this time.

Effect upon testicular morphology was identical to Nilevar and Norlutin, with the addition of hyalinization of the basement membrane of the seminiferous tubules appearing while still on treatment (see figs. 6–7). This phenomenon is seen during testosterone propionate and estrogen therapy, and was considered to be due to the higher amount of estrogen present in Enovid (1.5%), as contrasted with none for Nilevar and less than 0.2% for Norlutin.

Mechanism. Two factors militated against drawing immediate conclusions regarding gonadotrophin suppression: *a*) gonadotrophin titers at the pretreatment 12-hour aliquot level were too low to cause ovarian weight increases in the assay rats; *b*) when, therefore, uterine weight increases were used to interpret changes, it was discovered that not only pretreatment but also the therapy group and the control assay rats all had elevated uterine weights. It was subsequently discovered that the rat cages were contaminated with sufficient estrogen to elicit this degree of uterine stimulation. However during the two posttreatment gonadotrophin assays (at 16-hour aliquots) ovarian weight stimulation occurred.

Urinary estrogen excretion was elevated in each subject, and in the three subjects where enough extract was available for definitive assays, the estrogens rose from pretreatment levels of

0.65, 0.65 and 0.48 μg-equivalents to 124.0, 104.8 and 31.1 μg-equivalents of estradiol benzoate respectively.

Pregnanediol excretion did not increase, in fact appeared to decrease somewhat. Thus, as with Norlutin, the metabolic pathway of degradation is different than for progesterone. 17-ketosteroids, as with Nilevar and Norlutin, decreased.

Recovery phase. The first significant return of sperm in the seminal fluid occurred 11–14 weeks after discontinuing Enovid. A steady state of sperm output was reached at 23–24 weeks following cessation of medication with four subjects having higher counts and one lower counts than pretreatment. The hormonal levels were normal. Testicular morphology revealed incomplete recovery, and again revealed disorganization and increased numbers of germinal elements.

Final observations were made at 61, 71, 72 and 82 weeks after discontinuing treatment. In two men sperm counts were higher than, and in two the same as pretreatment levels. The morphological appearance of the testes in each instance was as good as, or better than, the pretreatment observations. Estrogens and gonadotrophins were normal.

'Pure' Enovid[6] and Ethynylestradiol-3-methyl Ether.[7] In order to separate the effects of Enovid from that of its estrogenic companion, two subjects were placed on 'pure' (less than 0.2% 3-MEE) Enovid, 30 mg daily orally in divided doses, and three subjects on the amount of the estrogen present in the regular daily dose of Enovid (i.e. 0.45 mg, administered orally 0.2 mg each morning and 0.25 mg each evening).

Sperm counts dropped to zero in each of the five subjects. The testicular morphology revealed hypoplasia and hyalinization of the basement membranes, and other changes similar to regular Enovid. Gonadotrophins dropped distinctly and unequivocally for each subject, and in each the estrogens were elevated. All developed gynecomastia.

Since both the 'pure' Enovid and the estrogen caused gonadotrophin suppression it would seem to be a safe assumption to infer that regular Enovid is also capable of complete suppression of gonadotrophins in normal men.

It seems apparent that the estrogen content alone can duplicate the effects of Enovid. A further conclusion is that 'pure' Enovid taken orally is converted to estrogen.

To ascertain whether the intramuscular route would avert the conversion of Enovid to estrogen, one subject was given 25 mg of pure Enovid once daily intramuscularly. Azoospermia, gynecomastia, loss of libido, potentia and a decrease in testicular size resulted within 8 weeks. Gonadotrophins dropped to undetectable levels, and estrogens rose approximately 75 times, which is more than can be accounted for by the minimal amount of 3-methyl ethynylestradiol present. Thus Enovid is converted to estrogen irrespective of the route of administration.

Progesterone. Although available for many years, progesterone has failed to find an acceptable role in clinical medicine. Our knowledge of its effect upon reproductive physiology in women is poorly understood, and well-documented information (other than its effect upon the endometrium) is only fragmentary. Reliable information is completely lacking concerning its effect upon human male reproductive physiology.

Perhaps it has no effect upon reproductive processes in men. If not, this seemed worthwhile knowing. Hence, five normal subjects, after preliminary observations, were given progesterone intramuscularly each day at the arbitrary dosage of 50 mg daily (arbitrary because that is the highest practical dosage currently available).

No immediate effects were noted, but after 4 weeks sperm counts began dropping, and by 10 weeks four of the subjects were azoospermatic and the fifth had dropped from an average of 72 to 5 million per ml. Libido and potentia reached minimal levels, slight gynecomastia occurred in two instances, and testicular size was reduced moderately. Testicular biopsy specimens were then obtained, as well as urine for hormonal assays. The azoospermia was first attained at *week-7* for one subject, *week-9* for two and *week-10* for one; at *week-9½*, one man still had 5 million sperm per ml.

Testicular morphology was impaired in each subject, but not nearly to the extent produced by a shorter duration of medication with the synthetic progestins. All elements of the germinal series could be identified, and for the most part were present abundantly. Leydig-cells, too, while somewhat altered, were present and identifiable. Very possibly longer exposure to progesterone administration may reveal greater change.

[6] Lot No. 938951.

[7] Lot No. 967811 (0.1 mg tabs.) and Lot No. 967812 (0.25 mg tabs.) kindly provided by G. D. Searle & Company, Chicago.

Note that changes with Nilevar were unequivocal in each case when given for 21–25 weeks, and in one case quite equivocal at 8 weeks.

Mechanism. Of the five, only two had detectable gonadotrophins, at the 12-hour aliquot level of assay, before treatment, whereas each of the five had discernible levels at the end of the medication interval when 16-hour aliquots were used. (Sixteen to twenty-four-hour aliquots were used at the end of the therapy period in this group and in all other groups.) Urinary estrogens exhibited no change, and 17-ketosteroids were reduced in each of four subjects, where observations were made. Similarly, pregnanediol excretion rose in each of four subjects tested.

Recovery phase. The first significant increase of sperm in the ejaculate was observed at 5 weeks in the man who had not quite reached azoospermia, and at 6, 7, 9 and 10 weeks respectively in the other four. Following discontinuance of the drug, sperm production reached a steady state by the 14th to 17th week, at which time two men had higher than, two equal to, and one somewhat lower than pretreatment levels. Testicular biopsy specimens now revealed the typical disorganization and increased cell mass which was observed during the same stage of recovery from the synthetic progestins. Hormone assay levels continued to be normal.

Final observations were made at 49 and 55 weeks after discontinuing injections in two subjects, since one was being paroled and the other being transferred to another penitentiary. In the others, final observations were made at 71, 81, and 85 weeks. The same sperm production plateaus continued, i.e. higher in two, the same in two, and lower in one man, who had developed bilateral chronic epididymitis. Biopsy specimens revealed that testicular architecture was as good as, or better than before treatment for each of the five in this group.

Subsequently, three other subjects were injected with 50 mg of progesterone per day, intramuscularly, for 90–111 days without significant reduction in sperm counts. In the one in whom a biopsy was obtained, no change (or improvement) in testicular spermatogenesis was noted. In a fourth subject, 17 - α - hydroxyprogesterone caproate, 125 mg per injection, was given intramuscularly 3 times weekly for 21 weeks and then increased to 125 mg daily for an additional 3 weeks. Sperm counts were reduced significantly, but did not reach azoospermia. The biopsy specimen obtained at the end of the injection period revealed that spermatogenesis was less active than that observed during the control period.

Our suspicion is that the dosage of progesterone used was at a threshold level, with some subjects responding more completely than others, and some not at all.

Hypothesis. One may hypothesize, therefore, that some of the steroids here tested, behaved in a conventional manner (Norlutin, Enovid and ethynylestradiol-3-methyl-ether) in seemingly suppressing spermatogenesis by suppressing gonadotrophins secretion, either through their intrinsic or converted estrogenicity. However, Nilevar, while suppressing spermatogenesis and markedly reducing gonadotrophins, did so without being estrogenic, either itself or through its metabolites. Finally, progesterone and hydroxyprogesterone caproate were neither estrogenic nor converted to estrogens, nor did they suppress gonadotrophins. Yet spermatogenesis was reduced, even if incompletely. Hence, the mechanism by which these steroids suppress spermatogenesis may be due to a direct effect upon the testis.

The authors wish to thank Rex Coy, Stephen W. Heller, Ted Roscoe, Shirley Lawrenson and Barbara Schmaeling for their diligent assistance and interest in these studies.

Most particularly, we wish to thank the subjects for their cooperation as volunteers; the many inmates of the Oregon State Prison Hospital who acted as surgical assistants, nurses, orderlies, laboratory assistants, and especially the two inmates who scheduled interviews, biopsies, supervised seminal fluid and urine collections, as well as administration of medications.

REFERENCES

1. Rock, J., G. Pincus and C. R. Garcia. *Recent Progress In Hormone Research.* New York: Acad. Press, 1957, vol. 13, p. 323.
2. Saunders, F. J. and F. A. Drill. *Endocrinology* 58: 567, 1956.
3. Landau, R. L., K. Lugibihl and D. F. Dimick. *Ann. New York Acad. Sc.* 71: 588, 1958.
4. Heller, C. G., W. M. Laidlaw, H. T. Harvey and W. O. Nelson. *Ann. New York Acad. Sc.* 71: p. 660 (Fig. 11), 1958.

DISCUSSION

WARREN O. NELSON

The Rockefeller Institute, New York City

THE WORK PRESENTED by Dr. Heller has been a collaborative study and as such has undergone considerable discussion between us. With most of Dr. Heller's remarks and interpretations I am in agreement but I do consider it important to point out two considerations where I believe the interpretations are open to question.

On several occasions Dr. Heller has mentioned 'increased numbers of germinal elements' or 'increased cell mass' in describing the events which occur during recovery of spermatogenesis from inhibition by the compounds used in the study. It is true that the spermatogenic process presents a disorganized and inferior appearance at that time, an appearance highly suggestive of the picture seen in testes during the puberal years and one in which the jumbled assemblage of germ cells might be viewed as totalling more than the expected normal numbers. However, this is an apparent and not a real increase due to the disorganized appearance provided by the sloughed cells. There is no objective basis for talking about 'increased numbers'.

In the case of the first 5 men who received progesterone the statement is made that the sperm counts dropped to zero in 4 subjects and were markedly reduced in the 5th. However, the posttreatment testicular biopsies failed to reveal any change of real consequence and thus serious doubt is thrown upon the accuracy of the seminal observations in this group of men. This point is strengthened by the absence of an effect of progesterone on the sperm counts in the second series of 3 men, a series undertaken because of concern about the accuracy of counts in the first series. In my opinion it is unlikely that progesterone in the dose used had any appreciable effect upon the testes.

Finally it may be said that studies in rats with the compounds mentioned here have shown that Enovid, Norlutin and ethynylestradiol-3-methyl ether are highly effective in suppressing spermatogenesis by way of gonadotrophic inhibition. Nilevar is less effective in the rat, but in this species the androgenicity of the compound is such that a direct stimulation of spermatogenesis occurs and overrides any gonadotrophic inhibition. Progesterone and 17-alpha-hydroxyprogesterone caproate failed to modify spermatogenesis.

USE OF NEW PROGESTATIONAL HORMONES IN GYNECOLOGY AND OBSTETRICS

A. E. RAKOFF

Department of Obstetrics and Gynecology, and Division of Endocrine and Cancer Research, Jefferson Medical College, Philadelphia, Pennsylvania

THE CLINICIAN now has available quite a variety of steroids with progesteronic activity for therapeutic purposes. These hormones are not all precisely the same in their biological actions and consequently they also differ in some of their therapeutic indications and side effects.

The progestestational steroids which are at present available on prescription include:

1) Progesterone; in oily solution for intramuscular injections, buccal tablets, and vaginal suppositories.

2) Ethisterone; (trade names: Pranone, Progestoral, Lutocylol, etc.) as tablets for oral use.

3) 17-α-hydroxyprogesterone caproate; (trade name: Delalutin), in oily solution for intramuscular injection.

4) Norethindrone (norethisterone); (trade name: Norlutin), as tablets.

5) Norethynodrel; (trade name: Enovid), as tablets, fortified with 1.5% ethynyl estradiol-3-methyl ether.

6) 17α-hydroxyprogesterone acetate; (trade name: Prodox), as tablets.

Numerous other related steroids are now known to have progesterone-like actions and are undergoing clinical investigation.

Progesterone, given with estrogen, will produce a typical secretory endometrium in the castrate and will maintain pregnancy in the human in whom the ovaries have been removed in early pregnancy. If given to the estrogen-primed women in full dosage, it will produce withdrawal bleeding in 4 days. This effect has been used to test for endogenous estrogen secretion and also as a clinical presumptive test for pregnancy if menstruation is delayed. The limitations and disadvantages of progesterone are its *a*) almost complete lack of effect when given orally, *b*) short duration of action and *c*) limited solubility. Progesterone may be given also in the form of buccal tablets or vaginal suppositories, but the absorption by these routes is uncertain and the method not always acceptable to the patient.

Hydroxyprogesterone caproate has the advantage of long duration of action and high solubility so that a single dose of 250 mg, given with estrogen if necessary, will simulate the action of the corpus luteum. For this reason it has proved useful and valuable in the treatment of gynecologic disorders. Hydroxyprogesterone caproate has also been used extensively in habitual abortion patients and although it is not yet proved whether it will maintain pregnancy in the oophorectomized pregnant woman, Allen and Wu (1) have found it to be effective in maintaining pregnancy in rabbits castrated about 24 hours after mating.

Although hydroxyprogesterone caproate has no oral activity, the acetate (Prodox), as well as certain other derivatives of hydroxyprogesterone, does have oral activity (2).

The 19-nor steroids with progestational activity, such as norethindrone and norethynodrel, have been of particular interest to the clinician because of their high oral potency, which appears to be of the order of five to ten times that of ethisterone in the human, their antigonadotrophic potency which permits easy inhibition of ovulation, their concomitant endogenous estrogenicity, their ability to produce rapid endometrial hemostasis, and the marked decidual reaction which they produce on the endometrial stroma. Disadvantages include their androgenicity, which is apparently minimal for the compounds now commercially available, but these together with ethisterone have recently been indited as possibly inducing virilization of the fetus in rare instances if given during pregnancy, and certain side reactions such as nausea, vomiting, bloating, breast pain, etc., which occurs in some patients on treatment. There is also question as to whether these preparations can maintain pregnancy in the progesterone deficient woman. They apparently do not maintain pregnancy in the rabbit (1, 3).

These various progesterone compounds may therefore be compared for therapeutic purposes on the basis of the following factors: *1*) qualitative

effects on the endometrium: *a*) glands, *b*) stroma, *c*) blood vessels; *2*) ability to inhibit ovulation, presumably by suppressing pituitary gonadotrophins; *3*) ability to maintain pregnancy: *a*) in the oophorectomized rabbit, *b*) in the progesterone-deficient human; *4*) endogenous estrogenicity; *5*) effective route of administration; *6*) relative potency; *7*) duration of action; *8*) side effects: *a*) androgenicity, *b*) anabolic effects, *c*) water retention, *d*) gastrointestinal disturbances, *e*) breast stimulation, *f*) effects on nervous system.

The chief advantages and disadvantages of these several compounds in the treatment of various clinical conditions will be discussed on the basis of the factors listed above.

TREATMENT OF MENSTRUAL DISORDERS

Progesteronic compounds are extensively used in the treatment of functional disorders of menstruation, particularly those resulting from pituitary-ovarian disturbances. Their efficacy however, is dependent upon the nature and persistence of underlying etiologic factors such as systemic disease, metabolic disturbances, psychogenic factors and extragonadal endocrinopathies.

Secondary Amenorrhea and Oligomenorrhea. If endogenous estrogen is present, as indicated by vaginal smears, progesterone given by intramuscular injection of 50 mg on 2 consecutive days, will generally induce bleeding about 4 days after the last injection, and is therefore the preparation of choice if one wishes to induce prompt bleeding. This response to progesterone may therefore also be used as a clinical test for adequate estrogen secretion (4). Hydroxyprogesterone caproate in a dosage of 250 mg in a single intramuscular injection will induce bleeding in estrogen-primed women in approximately 12 days (± 2 days) from a fairly normal late secretory endometrium, thus simulating the normal corpus luteum. Bleeding can also be induced in such instances by the daily administration for 10 days of ethisterone 50 mg, norethindrone or norethynodrel 10 mg, or acetoxyprogesterone 50 to 100 mg. The endometria in patients treated with norethindrone and norethynodrel are atypical, showing relatively greater stromal than glandular stimulation, as demonstrated particularly by Pincus, Rock and Garcia (5). These irregularities are much more marked in patients receiving therapy for 20 days as compared to those treated for 10 days. Greenblatt (6) has presented evidence suggesting that this is not a true qualitative difference but rather a quantitative expression of the high progesteronic potency of these compounds. There are also some histochemical differences in the secretory endometria produced by the 19-nor steroids, particularly in the distribution of glycogen, alkaline phosphatase and lipids, as shown by Shah, Long and Southam (7), but these too may represent a quantitative rather than a qualitative difference.

A distinct difference in the reaction of these compounds is seen in patients who have little or no endogenous estrogen. Bleeding of varying amounts will generally follow the administration of norethindrone and norethynodrel because of their endogenous estrogen effect (supplemented with additional estrogen in the case of norethynodrel as Enovid), whereas estrogen must be given with the other compounds, either beforehand to prime the endometrium or it may be added concomitantly.

In clinical practice this type of treatment is usually given for 3 consecutive months and then stopped to determine whether spontaneous cycling will be resumed. If subsequent spontaneous function is not initiated then the patient should be re-evaluated with regard to the nature of the underlying etiologic factor before resuming further substitutional therapy.

Dysfunctional Uterine Bleeding. The 19-nor steroids have been particularly useful in the control of hypermenorrhea and polymenorrhea of dysfunction origin (4). Excessive bleeding can generally be stopped within 24 to 48 hours by the administration of 10 mg orally every 4 to 6 hours. When bleeding has ceased, the dosage is gradually decreased to 10 to 20 mg daily for a total of 3 weeks. The flow which follows is usually fairly normal. The medication is then given in a dosage of 10 mg daily for 20 days during the next three cycles and then stopped to see if normal cycling will resume. If breakthrough bleeding occurs at any point, the dosage is increased to 20 mg daily.

With hydroxyprogesterone caproate, treatment may be initiated by giving 500 mg by intramuscular injection. This will usually cause decrease or cessation of bleeding in a few days followed by a regular flow about 10 days later from a secretory type of endometrium, thus providing a so-called 'medical curettage'. Cyclic therapy is then instituted, such as by the administration of 10 mg of estradiol valerate and then one week later 10 mg of estradiol valerate plus 250 mg of hy-

droxyprogesterone caproate. This type of cyclic therapy is continued for three months. It should be emphasized that it is often more difficult to obtain initial hemostasis with hydroxyprogesterone caproate than with the 19-nor steroids. Progesterone itself by intramuscular or buccal route is usually not at all satisfactory for the immediate control of dysfunctional bleeding.

DYSMENORRHEA

Severe primary dysmenorrhea is well known to be alleviated by inhibition of ovulation. This may be accomplished by the oral administration of an estrogen in sufficient dosage, given from *day* 5–25 of the cycle, or by the administration of the 19-nor steroids in a dosage of 10 mg for the same period of time. The latter compounds have the advantage of less often being associated with ovulation and subsequent menstrual irregularity. On the other hand, in some patients relief of pain is not obtained or at least not as adequately as with estrogen alone. This again raises the often discussed question as to whether bleeding from a secretory type of endometrium is more likely to be associated with pain, even if ovulation is inhibited.

ANOVULATORY INFERTILITY

Progesterone and ethisterone have been extensively used for the treatment of infertility due to anovulation or an inadequate corpus luteum phase. Despite its effectiveness in a varying proportion of cases, the modus operandi remains obscure or controversial. Among the suggested explanations are *a*) production of a more adequate nidatory endometrium, *b*) inhibition of gonadotrophins with rebound to higher levels in subsequent cycles, *c*) release of conjectural biochemical substances from a secretory endometrium which stimulate better pituitary response in the next cycle. Somewhat similar results have been obtained with the newer progestational compounds. Several 'plans' of treatment have been suggested for the 19-nor steroids: *1*) administration from *day 5–day 25* for 3 cycles, and then withdrawal of therapy; *2*) administration for 10 days beginning about *day 16* of the cycle; *3*) administration beginning on *day 16*, or about 2 days after the expected day of ovulation, and continuing until it is known that the patient is not pregnant, and then stopping treatment to allow bleeding; or continuing treatment if she is known to be pregnant (8).

Hydroxyprogesterone caproate is generally given in a dosage of 250 mg by injection on *day 16*, with or without added estradiol valerate, depending upon the estrogen status of the patient. Pregnancy has resulted in anovulatory patients with all of these hormones. From our own experience I have not been able to note that any one of these agents is more efficacious in this respect than the others.

ENDOMETRIOSIS

The continuous administration of the 19-nor steroids produces a pseudo-pregnancy state, characterized by amenorrhea and decidual transformation of the endometrium. In patients with endometriosis, Kistner (9) has found that the reaction maintained over a period of months leads to relief of pain and regression of the endometriomas, which he attributes to decidual necrosis and absorption of functioning endometrial tissue. This therapy has been used as a primary medical treatment for this condition or as preoperative therapy or as follow-up therapy following conservative surgical treatment. Treatment may be started at a dosage of 10 mg daily and then gradually increased at intervals of 2 weeks to a maintenance dosage of 20 to 40 mg daily for a period of 6 months or more. The purpose of increasing the dosage is not only to obtain better regression of the endometriosis but also to prevent or control breakthrough bleeding. In patients with extensive adenomyosis there may be persistence of pain. Kistner has recently reviewed his extensive experience and those of others with this type of therapy and has found the results to be quite gratifying.

THREATENED AND HABITUAL ABORTION

Progesterone with or without estrogen has been purported to be of value in the treatment of some cases of threatened and habitual abortion, presumably those with a deficiency of the corpus luteum or a placental deficiency of these hormones. Reifenstein (10) has recently reviewed the results of treatment of habitual aborters by a large number of investigators using hydroxyprogesterone caproate. These data indicated that in the dosage of 375 to 500 mg once a week, the fetal salvage is comparable to that obtained by other methods of treatment.

Preliminary results (table 1) which we have obtained with the 19-nor steroids in a small group of patients with habitual or threatened abortion,

TABLE 1. RESULTS OF TREATMENT WITH ENOVID IN HABITUAL AND THREATENED ABORTION

	Number of patients	Number of previous pregnancies	Number of living babies	Previous fetal salvage rate	Salvage rate in current pregnancy
Total group	38	130	59		
Three or more consecutive abortions	18	82	23	28%	14 infants or 77%
A. Primary habitual aborters*	6	20	0	0	4 infants or 66%
B. Secondary habitual aborters†	12	62	23	37%	10 infants or 83%
Threatened abortion	20	48	36	75%	12 infants or 60%

* Three or more abortions, no live births.
† One or more live births followed by 3 or more abortions.

were similar to those which we have obtained with other progesterone compounds. Treatment was started with a dosage of 10 mg daily during the first month of gestation and increased to 20 mg thereafter. In threatened abortion, dosages of 20 to 40 mg daily were used. On the other hand, Seidl, Epstein and Kupperman (11) have noted poor results in a group of 26 patients. Their study was limited to women with low pregnanediol levels. Particular care must be exercised to detect missed abortions during therapy with these potent progesteronic compounds, since they frequently prevent telltale bleeding. Quantitative assays on the serum for chorionic gonadotrophin are very helpful in following the fate of the pregnancy. A falling titer in early pregnancy is usually indicative of death of the embryo, except in instances where there is molar degeneration. In the latter circumstance the titer may rise again after an initial fall.

SIDE REACTIONS AND UNTOWARD EFFECTS

A discussion of the side effects, particularly those which are undesirable or harmful is particularly important for consideration by this panel.

Progesterone itself is a remarkably well-tolerated hormone. It does not often produce any untoward local or systemic effects. In a concentration of 50 mg per cc it will on rare occasions produce local pain, irritation or formation of oily cysts which may require subsequent excision. Exacerbation of unusual menstrual molimina may also occur after any of the progesterone compounds in rare patients who appear to have an endogenous sensitivity to progesterone.

Some patients appear to develop fluid retention during progesterone administration, particularly when it is given together with estrogen. On the other hand, it has been shown by metabolic studies carried out by Landau and his associates (12), that the more common action of progesterone is to induce diuresis with an accompanying loss of sodium and chloride. It is probable that the pretreatment hormonal status of the patient influences this response to progesterone.

In women who have received progesterone therapy in cyclic fashion or even continuously for many months, there is no evidence of subsequent organic change or functional impairment in the pituitary or ovaries. Normal cycling and subsequent pregnancy appears to occur promptly after withdrawal of treatment. I am not aware of any evidence of carcinogenic or other neoplastic effects on the uterus.

Some growth, swelling and tenderness of the breasts is often noted during the course of treatment with various progestational agents, more often with the 19-nor compounds than with the others. This is generally not sufficiently marked to be annoying and in small breasted women it may often be appreciated. The breast changes generally regress completely after therapy is discontinued.

Gastrointestinal disturbances are a troublesome side reaction with some of the 19-nor steroids. It occurs more frequently with Enovid than with Norlutin, and may in some patients cause severe nausea and vomiting. It seems likely that the added estrogen in the form of 1.5% of ethynyl estradiol-3-methyl ether is in part at least responsible for the gastrointestinal symptoms caused by Enovid. In most instances, these symptoms will gradually diminish after a few days. Their intensity can also often be alleviated by taking the medication after a meal or with a glass of milk, particularly at bedtime, or with an added anti-emetic drug. In some instances, however, the medication has to be discontinued because of the persistence of the nausea and vomiting.

Other occasional symptoms sometimes noted are bloating, vertigo, dyspnea, leg cramps,

headache and various nervous manifestations akin to those noted with premenstrual tension. In some women there is a marked gain in weight. It has long been known that progesterone exerts certain androgenic effects in some species, as for example, the prevention of castration effects on the prostate and seminal vesicles of rodents. When given to women even over a prolonged period of time in high dosage, there have usually been no evidences of virilization. This has likewise been true of ethisterone. Experience with hydroxyprogesterone caproate also indicates that it is quite free of virilizing effects.

With the advent of the new nor-testosterone preparations there has been a sharp lookout by the clinician for masculinizing effects, particularly since some of the analogues tested were found to be somewhat androgenic both in animals and in women, but norethindrone and norethynodrel in the usual therapeutic dosages have not generally caused these effects. There have been a few rare instances in which women on high dosage have developed some deepening of the voice or some slight increase in hirsutism, but such cases have been rare indeed.

An alarm has been sounded however by Wilkins and his associates (13, 14), by Grumbach (15) and others, with regard to the possible induction of virilization of the female fetus in mothers treated with various progesterone compounds not only of the 19-nor series, but particularly with ethisterone and in a few cases even with progesterone itself. These babies have generally shown clitoral hypertrophy of varying degrees. In several instances there has been partial fusion of the labio-scrotal folds. It would appear that this type of female pseudo-hermaphroditism requires the administration of androgenic substances prior to the 3rd month of gestation. The upper genital tract and gonads are not affected. It is obvious that these virilizing effects on the fetus occur in only very rare instances. Indeed, it is only recently that such reports have appeared despite the fact that progesterone and ethisterone have been given to countless thousands of pregnant women over the past 2 to 3 decades. In many hundreds of women treated with fairly large dosages of progesterone or with ethisterone, I have not seen a single bonafide case. Recently I noted slight clitoral enlargement in one premature infant of a habitual aborter treated throughout pregnancy with Enovid. Interestingly enough Bongiovanni (16) has recently reported 4 cases in women treated with diethylstilbestrol.

Since clitoral hypertrophy or marked types of female pseudo-hermaphroditism may occur spontaneously and since very few cases are reported in women treated with progestogens during pregnancy, there is still some question of a causal relationship; and if there is an etiologic relationship, the reason for the extremely low incidence is puzzling. The obstetrician and the pediatrician should now pay close attention to these babies, and the obstetrician will perhaps be more rigid in his indication for the administration of these hormones to pregnant women.

REFERENCES

1. Allen, W. M. and D. H. Wu. Abstracts XV Annual Meeting, American Society for the Study of Sterility, April, 1959.
2. Davis, M. E. and G. L. Wied. *J. Clin. Endocrinol.* 17: 1234, 1957.
3. Saunders, F. J. and V. A. Drill. *Ann. New York Acad. Sc.* 71: 516, 1958.
4. Southam, A. *Ann. New York Acad. Sc.* 71: 666, 1958.
5. Pincus, G., J. Rock and C. R. Garcia. *Ann. New York Acad. Sc.* 71: 677, 1958.
6. Greenblatt, R. B. *Proceedings of a Symposium on Enovid.* Skokie, Ill.: G. D. Searle Co., Nov. 25, 1958.
7. Shah, P. N., M. E. Long and A. L. Southam. *Ann. New York Acad. Sc.* 71: 617, 1958.
8. Tyler, E. T. and H. J. Olson. *J.A.M.A.* 169: 1843, 1959.
9. Kistner, R. W. Abstracts XV Annual Meeting, American Society for the Study of Sterility, April, 1959.
10. Reifenstein, E. C., Jr. *Ann. New York Acad. Sc.* 71: 762, 1958.
11. Seidl, J. E., J. A. Epstein and H. S. Kupperman. *Internat. Fertil.* 3: 349, 1958.
12. Landau, R. L., K. Lugibihl and D. F. Dimick. *Ann. New York Acad. Sc.* 71: 588, 1958.
13. Wilkins, L., H. W. Jones, Jr., G. H. Holman and R. S. Stempfel, Jr. *J. Clin. Endocrinol.* 18: 559, 1958.
14. Jones, H. W, Jr. and L. Wilkins. Abstracts XV Annual Meeting, American Society for the Study of Sterility, April, 1959.
15. Grumbach, M. M. Abstracts XV Annual Meeting, American Society for the Study of Sterility, April, 1959.
16. Bongiovanni, A. M. Abstracts XV Annual Meeting, American Society for the Study of Sterility, April 1959.

DISCUSSION[1]

HERBERT S. KUPPERMAN

New York University-Bellevue Medical Center, New York City

THERE IS LITTLE that one can add to the complete and thorough review which has been presented in such an able and competent manner by Dr. Rakoff. However, it would be well worth-while to mention some of our own experiences with the use of the newer progestational steroids depicting their mechanism of action and enlarging upon some of the points raised by Dr. Rakoff.

The treatment of functional uterine bleeding with these preparations has been most effective in producing relatively complete desquamation of the endometrium, particularly in those patients with cystic glandular hyperplasia or anovulatory cycles. The degree of endometrial desquamation occurring is dependent upon the extent of stromal changes that are induced by the progestational steroid. Since a marked decidua-like reaction occurs in the stroma following the use of the 19-norsteroids, the desquamation achieved following such therapy may be more complete than that noted after progesterone itself. Of all the progestational steroids studied after oral administration we have found that 140 mg of norethindrone or norethynodrel will produce a predictable effect when administered over a period of 7–14 days. We have not found smaller doses to be as consistent in inducing an adequate effect upon the stroma or glands of the endometrium. Acetoxyprogesterone in doses of 500 and 700 mg over the same period produced secretory endometrium in 80% of the patients so treated. 6-methyl-acetoxyprogesterone was much more efficacious than the parent compound and induced a secretory endometrium in doses of 25–30 mg ingested over the same period of time. Norethandrolone usually required 210 mg to accomplish a comparable effect upon the endometrium. The androgenic potential of this latter compound in doses of 30–40 mg per day may make this preparation the one of choice in patients with active bleeding at the time therapy is instituted. The use of the 19-norsteroid compounds in patients bleeding excessively at the time the steroids are administered, as a rule, is not followed with as prompt a cessation of bleeding as was observed when concomitant androgen therapy was used parenterally with progesterone. The role of androgens in inducing hemostasis cannot be equalled by the use of the progestational steroids alone except for those that show a significant androgenic effect.

The treatment of dysmenorrhea with the 19-norsteroids may be accomplished very adequately by the administration of 10 mg of either norethynodrel or norethindrone twice a day for 8 days, starting on the 10th day of the menstrual cycle. This offers the patient a relatively short time for drug administration and will inhibit ovulation regardless of how many consecutive cycles such therapy is employed. This is unlike the results obtained with estrogen when higher doses of the estrogen must be employed in subsequent cycles to maintain the patient free of pain.

We have been very impressed with the beneficial results achieved with the 19-norsteroids in the treatment of endometriosis and have confirmed the observations so well documented by Kistner (1). Our therapeutic approach, however, differs somewhat and more recently has been modified to the extent that we have used a combination of both norethynodrel and norethindrone. The purpose of the combination is to obviate the undesirable side effects that have been noted when appropriate doses of either one of the 19-nor compounds are used alone. In contrast to the procedure requiring a gradual increase in dose of steroid necessary to suppress menses and thereby effectively control endometriosis we initially start with a 30 mg dose per day of either one of the steroids. This procedure has proved to be most satisfactory and will usually prevent breakthrough bleeding in the majority of cases. If breakthrough bleeding does occur, the dose of the steroid is increased by increments of 10 mg per day and maintained at this level for at least 5 days. If bleeding still occurs the dose is increased again by 10 mg per day. If menses cease then the dose of steroid achieving this is maintained for a total duration of 6–9 months or until bleeding occurs again. Approximately one-fourth of our patients required doses exceeding the initial 30 mg per day. Either norethynodrel or norethindrone have produced some undesirable side effects in doses ranging between 30–50 mg per day. The side effects differ according to the particular steroid being used. When norethynodrel is employed alone some patients have noted an increased degree of breast engorgement which may

[1] The investigative work reported in this discussion has been supported in part by Grant RG 5162 (c1), U. S. Public Health Service.

or may not be accompanied by nausea and vomiting. On the other hand, nausea and vomiting may be the only presenting complaint. This may be partially alleviated by ingestion of milk with the medication and/or by the use of perphenazine suppositories. When nausea and vomiting do occur, regardless of procedure used to combat it, it will usually subside after 7–10 days of continuous treatment in much the same manner noted after stilbestrol therapy. The nausea-inducing effect of norethynodrel may be attributed to a central phenomenon rather than due to a local irritating phenomenon. In contrast, patients on similar doses of norethindrone, while not complaining of nausea and vomiting or breast engorgement, have occasionally noted some increase in hirsutism and facial acne. In order to diminish the undesirable side effects of either steroid without altering their efficacy, we have simultaneously administered combined doses of 15 mg of each steroid. There has been a decreased incidence of gastrointestinal disturbance, amelioration of breast engorgement, and little adverse effect upon acne or hair growth with such combined therapy. Regardless of the type of therapy employed, the steroids should be continued for periods of time ranging from 6–9 months, maintaining a state of amenorrhea during this period of time. Resumption of normal ovarian function occurs promptly after the steroids are discontinued. Histologically the endometrium obtained after 6–9 months of therapy revealed marked edema of the stroma associated with almost complete suppression of glandular activity (fig. 1). In contrast, 2–3 weeks after the suppressive doses of steroids are discontinued one may note resumption of usual cyclic morphologic patterns (note the excellent proliferative phase

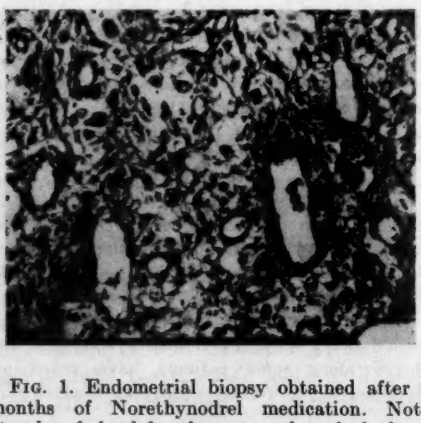


FIG. 1. Endometrial biopsy obtained after 6 months of Norethynodrel medication. Note atrophy of glandular elements and marked edema of stroma.

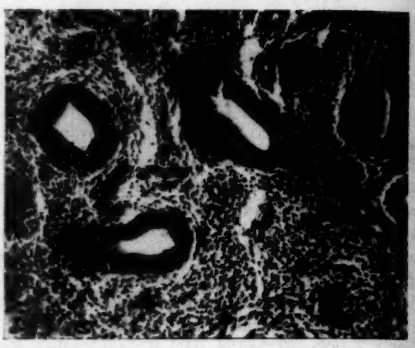


FIG. 2. Endometrial biopsy taken 1 month after discontinuing Norethynodrel therapy. Note normal proliferative phase activity with normal stroma.

and glandular activity one month after discontinuing therapy—fig. 2). Words of caution and trepidation have been raised by those who have not used the steroids, but who feel that caution or therapeutic nihilism are the order of the land and that suppression of ovarian function may have a deleterious effect upon future activity of the ovary. While this is an ever-present theoretical potential, we must not lose sight of the fact that nature has provided us with an excellent example of how suppression of ovarian function, even from the prenatal period on, has not resulted in adverse effects upon subsequent ovarian function when the inhibitory effects of the steroids are negated. Patients with the congenital adrenogenital syndrome, with complete suppression of their gonadotrophin secretion by the increased production of androgenic precursors from their adrenal cortex, respond promptly and dramatically to corticoid therapy as soon as the excessive secretion of the androgen steroids is diminished and its inhibiting effect upon gonadotrophin function is held in abeyance. Such individuals began to menstruate and ovulate shortly after initiation of corticoid therapy, even though ovarian function had been suppressed for some 10, 20 or even 30 years. These findings exemplify very vividly the fact that suppression of gonadotrophin secretion by steroidal agents would conceivably not be associated with adverse effects upon subsequent ovarian function when the steroids are no longer administered. Pincus and his group have shown that when norethynodrel has been administered in doses sufficient to prevent ovulation and conception, cessation of steroid therapy is followed by normal ovarian function as evidenced by pregnancy which has occurred immediately after the steroid has been discontinued in many of his patients. In

TABLE 1. EFFECT OF PROGESTATIONAL STEROIDS UPON PREGNANCY SALVAGE

Oral Therapy	No. of Patients Treated	No. of Term Deliveries	Viable Prematures	Abortions on Therapy	No Follow-up	Patients Still Under Treatment
Progesterone 1000 mg o.d.	85	48 Fetal salvage = 54/73 (74%)	4 (2)	16 Abortions = 17/73 (23%)	1	14
19-Nortestosterone compounds	22	5 Fetal salvage = 7/22 (32%)	2 (2)	13 Abortions = 13/22 (59%)	0	0
Acetoxyprogesterone 200 mg o.d.	18	8 Fetal salvage = 8/13 (62%)	0	5 Abortions = 5/13 (38%)	4	4

Figures in parentheses = one obstetrical accident and one non-viable premature.

addition, one should note that the complete suppression of ovarian activity noted during the 2nd and 3rd trimester of pregnancy is followed by resumption of normal and cyclic ovarian function soon after the secretion of suppressive steroids from the placenta has been nullified by the passage of the products of conception at parturition.

Progestational steroids achieve their most gratifying use in the successful treatment of patients who have had a history of repeated spontaneous abortions. While many other agents in uncontrolled studies have been used, the results at times have been indifferent or unusually successful, depending upon the optimistic or pessimistic nature of the investigator. In contrast it is important to note that when adequate controls are set up and definite prerequisites delineated the likelihood of producing a beneficial effect with progesterone in progesterone-deficient individuals is good. The results of our studies are presented in the accompanying table and show that the use of certain progestational steroids, particularly progesterone, have been effective in preventing the occurrence of repeated abortions in patients with a history of two or more repeated abortions and in whom a low pregnanediol for their phase of pregnancy, at the time therapy is initiated, has been demonstrated. Normally in our laboratories the pregnanediol excretion is usually 10 mg per month of pregnancy per 24 hours. In the series of patients presented in the accompanying table we note that progesterone given in doses of 1000 mg per day orally has been effective in preventing a recurrence of the spontaneous abortion noted in previous pregnancies. On the other hand, we have found the 19-norsteroids to be ineffective in significantly altering the course of events in patients with a history of repeated abortions. It is interesting to note that with the doses of progesterone employed there have not been any associated adverse effects upon the external genitalia of the fetus born of mothers receiving such medication. As a matter of fact, the only abnormality of genitalia noted in our entire series was that of hypospadias in a male infant. This finding is just opposite to that anticipated from the results described in the literature.

One word about the possible abnormal external genitalia which may be observed in infants born of mothers placed upon ethisterone, norethisterone and progestational steroids in general. The use of these compounds may be associated with a potential fetal abnormality in that the external genitalia of female fetus may show partial or complete labial fusion and significant clitoral enlargement. However, in view of the recent description by Bongiovanni *et al.*[2] of 3 infants (born of mothers receiving stilbestrol) who showed the precise abnormal external genitalia described by Wilkins *et al.* (2), one should reconsider the exact role of the progestational steroids in producing these abnormalities. Is it not possible that factors completely divorced from the administration of the exogenous steroids may be important in producing the congenital abnormalities consisting of enlargement of the clitoris with partial, or complete fusion of the labia? We have in our own files cases of several children born of mothers who have never received any steroidal hormone therapy during their pregnancy, showing the very same picture described by Wilkins *et al.* and Bongiovanni (figs. 3, 4). Such findings should raise some question of doubt in the minds of those who feel that exogenous steroids play the primary role in the etiology of the gross abnormalities of the external genitalia in the newborn female. In addition, tremendous doses of stilbestrol have been used in the past for the treatment of habitual abortion without any apparent fetal abnormalities being noted by the investigators who employed such medication. Consequently, one could antici-

[2] Bongiovanni, Alfred M. The use of steroids during pregnancy from the viewpoint of the pediatrician. Presented before the April, 1959 meeting of the American Society for the Study of Sterility.

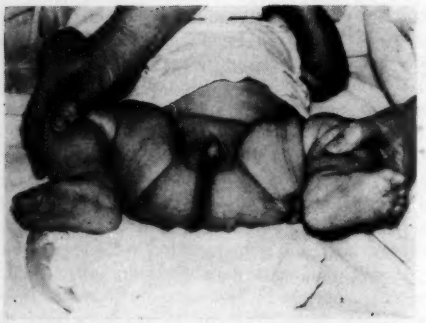


FIG. 3. Patient L.B., 2-year-old female infant. Mother never received hormone therapy during pregnancy. Chromatin pattern positive. 17 KS and Pregnanetriol normal.

FIG. 4. Patient J. W., 3-month-old female infant with positive chromatin pattern and normal 17 KS and Pregnanetriol secretion. Mother never received any hormonal therapy during pregnancy.

pate that several of the infants with abnormal genitalia delivered from mothers on steroids, might have shown the same type of abnormality noted on the basis of coincidence alone.

The management of children with this type of abnormal external genitalia demands comment. Many of the children with minimal clitoral enlargement without fusion of the labia will probably not require plastic surgery inasmuch as the clitoris will gradually diminish in size. In contrast, complete labial fusion with an enormously enlarged clitoris, would require a plastic procedure early in the life of such an infant who has normal steroid excretion and a positive chromatin pattern. Surgery should be of the type where the clitoris is not completely amputated.

Another point to be emphasized is that those infants showing the abnormalities of external genitalia described above must be adequately diagnosed before such anomalies can be specifically attributed to the steroids per se which the mothers received. It is essential that chromatin studies be obtained, as well as determination of the level of 17-ketosteroids and pregnanetriol in a 24-hour specimen. One must not assume that abnormalities of external genitalia, of infants delivered from mothers on steroid therapy, are due to the steroid administered to the mother unless the possibility of congenital adrenogenital syndrome or intersex have been ruled out by the above studies.

It would be wise to emphasize that if the physician feels that the use of the steroids described above, despite their ever-present potential effect of masculinization of the external genitalia in the fetus, will permit a viable birth to take place, their use then is not contraindicated. The use of frank androgens, such as methyltestosterone or methylandrostenediol, has no place in the treatment of the pregnant female whether for the purpose of preventing a spontaneous abortion or for the amelioration of any endocrinopathy or for treating a neoplasm. Androgens must be considered as unphysiologic therapy early in pregnancy and have no role in the control of uterine bleeding at this time.

The physician and the gynecologist, in particular, now have available a new group of compounds which show some of the characteristics of progesterone. They are by far the most potent oral progestational steroids available to date. While they differ from progesterone in their specific effects upon the endometrium, in that they cause greater stromal hyperplasia with low secretory or glandular activity, this may be of advantage in the treatment of the different endocrinopathies with which the physician is confronted. Their promiscuous use in early pregnancy may be associated with abnormalities of the external genitalia of female infants born of mothers so treated. These effects, however, would not be anticipated to have any permanent damage. As with any new medication available to the physician, certain difficult disease entities which had been refractory to therapy in the past, now may be treated effectively. However, in so doing newer complications due to drug therapy may appear. The search for more specific progesterone compounds goes on. Consequently, one may anticipate that eventually there will be made available and within the economic range of all patients, a compound having all the attributes of progesterone with few of its adverse effects.

REFERENCES

1. KISTNER, ROBERT W. *Am. J. Obst. & Gynec.* 75: 264–278, 1958.
2. WILKINS, LAWSON, H. W. JONES, G. H. HOLMAN AND R. S. STEMPFEL, JR. *J. Clin. Endocrinol.* 18: 559–585, 1958.

AMERICAN INSTITUTE OF NUTRITION

Symposium on Effects of High Calcium Intakes[1]

Chairman: OLAF MICKELSEN

INTRODUCTION

OLAF MICKELSEN

National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

ON BEHALF OF the Metabolism and Nutrition Study Section of the Division of Research Grants of the National Institutes of Health and the American Institute of Nutrition, we welcome you to this symposium on 'The Effects of High Calcium Intakes.' Sponsoring symposia is one of the less well-known functions of the Division of Research Grants. Most of you have become familiar with that Division through requests for support of your research projects. The latter represents the major activity of that Division.

Occasionally the Division sponsors a symposium to explore an area of biological science that might profitably receive more emphasis research-wise or to evaluate the data existing in an area that is subject to considerable confusion. Three years ago, in Atlantic City during the Federation meetings, the Metabolism and Nutrition Study Section sponsored a symposium on the interpretation of data secured with isotopically labeled proteins of biological significance. The papers presented at that meeting appeared as a supplement to the July, 1957, issue of the Federation Proceedings.

Today's symposium was planned by a committee consisting of Drs. Smith Freeman, M. K. Horwitt, George Davis and the speaker. Dr. Davis also appears on the program as a speaker in recognition of his many contributions to the field of mineral metabolism.

Our primary purpose in organizing the symposium was to collect and to evaluate the available data as they pertain to the effects of high calcium intakes. This obviously raises the question "How much is 'high?' " For the purposes of our discussion we shall consider anything above one gram of calcium per day high for human beings. One gm of calcium is the approximate average value suggested for adults and adolescent children in the Food and Nutrition Board's 'Recommended Dietary Allowances' (1).

During the past few years there has been considerable debate among those interested in nutrition as to whether intakes of calcium at or slightly above the levels listed in the 'Recommended Dietary Allowances' might be harmful. The physiological dangers that might result from such calcium intakes have not been defined.

For this symposium, we thought it desirable to secure some indication of the calcium intake in the United States in order to set the problem in its proper perspective. Obviously, the calcium intake is only one aspect of the problem that must be considered. Although the presence of large amounts of calcium in the gastrointestinal tract may have profound effects on the body, most of the present-day fears about high calcium intakes, insofar as human beings are concerned, have been limited to the calcium that was absorbed. For this reason we shall next consider the factors that influence the absorption of calcium. Once the calcium has been absorbed, it might affect a number of tissues and organs. One of the first of these to come to mind is the genito-urinary system. Since the kidneys are the first point of attack in that system, we shall review

[1] Sponsored by the Metabolism and Nutrition Study Section, Division of Research Grants, National Institutes of Health, and the American Institute of Nutrition, Atlantic City, New Jersey, April 14, 1959.

what is known about the actions of high calcium intakes on renal function. By implication and suggestion, the primary emphasis in so far as high calcium intakes is concerned has been on the formation of renal calculi. We shall explore this subject both as it pertains to animals and to man. The effect of high calcium intakes on blood, bone and soft tissues will also receive some consideration. The last area to be explored is the effect of high intakes of calcium on the absorption of other nutrients.

In developing this symposium, the committee recognized that individuals can be maintained in calcium balances at levels of intake that are considerably below those that are to be considered here. Furthermore, since calcium balances can be established over a wide range of dietary intakes, it is likely that the minimal calcium requirement may also be below any of these levels. It was deemed advisable to emphasize the fact that these two areas are beyond the scope of our present deliberations.

With the preceding as a frame of reference, let us proceed to the papers according to the program. When the papers have been presented, there will be a formal discussion. But first let me, on behalf of the Metabolism and Nutrition Study Section, express our sincere gratitude to the speakers for the time and effort they so generously have given to this symposium.

REFERENCE

1. *Recommended Dietary Allowances*. Pub. No. 589, Nat. Research Council (U.S.), 1958.

CALCIUM INTAKE OF CHILDREN AND ADULTS

MARGARET A. OHLSON AND GENEVIEVE STEARNS

Departments of Internal Medicine and Orthopedic Surgery, State University of Iowa College of Medicine, Iowa City, Iowa

FASHIONS EXIST in nutrition just as do vogues in medicine and in women's hats. During the years of discovery of the vitamins, and of the role of minerals and amino acids, attention was centered on a lack of these substances. In an expanding economy with ample food supplies and the development of concentrated packages of nutrients, the 'luxus consumption' principle (1) is an attractive one. Pressures of advertising are added to the voice of the nutritionist for the consumption of just a little more. It was inevitable that the pendulum eventually should swing in the opposite direction.

More knowledge of the food supplies of other societies, emphasis on the number of overweight adults in our society and the high death rate of young adult men from cardiovascular disease focused attention on the role of excess food and immediately the call for austerity in food intake was sounded. In the case of calcium, the association of calcium in food, particularly in milk, with the products of radioactive fall-out has added to the demands for reduction of our standards of intake. In this case, as in most vogues, there probably is a happy medium somewhere between the concept of minimum requirement for survival and luxury intakes of nutrients. It is pertinent to review the known facts of calcium as a nutrient and attempt to evaluate these facts from the point of view of what is a high calcium intake.

This paper will be limited to a discussion of the calcium intakes of various population groups as reported in the literature since 1942 and to a number of factors known to influence the utilization of this mineral under the conditions of living and eating of the average citizen.

In any attempt to measure food intake there is always an interplay between experimenter and subject which introduces a degree of bias into measurement of food consumption. For instance, it was demonstrated ten years ago in a study of active homemakers that a 24-hour recall diet seemed to measure higher calorie intakes than weighed diets when these techniques were applied to the same group of subjects (1a). Weight losses during 10 days on a weighed diet confirmed the impression that between-meal eating was not seen when each mouthful needed to be weighed. Thus the determination of food intakes may be approached by a number of experimental tech-

niques all of which can be expected to yield slightly different results, and the absolute accuracy of such studies, in terms of the degree to which the data reflect day by day practice, is relative. This should not invalidate carefully conducted studies but should alert the investigator to accept the results of other experimental approaches as being equally important and valid for the method used.

The classical surveys of the Department of Commerce (2) and, more recently, the U. S. Department of Agriculture (3), related the movement of food supplies into retail channels during the last 100 years. These studies report an increase in the calcium available in the food supply since 1935. The more recent surveys of the Human Nutrition Research Division (4–7) of the U. S. Department of Agriculture represent inventories of family purchases. In spite of an increase in national supplies, family inventory studies such as those of Phipard (8), Ohlson *et al.* (9), found calcium to be the nutrient most often provided in the family purchase of food at quantities less than those recommended by the Food and Nutrition Board (10). The single most important source of calcium in diets in the United States was milk or its products, the percentage of calcium obtained from milk increasing as milk intake increased.

Many survey studies are reported in which individual, self-chosen food intakes have been recorded and estimates of the nutritive values of food consumed made from tables of food composition. These studies have differed widely in methodology, period of observation, and combinations of data which have been made for reporting purposes. In the studies selected for presentation, individual food intakes were computed from a dietary history, from food as eaten or, in retrospect, as a 24-hour recall.

All studies included represented presumably healthy population samples in the United States and were reported in terms which could be combined and compared (11–51). Table 1 gives the number of individuals studied and age groupings used. Boys and girls have been combined through age 12. There is a concentration of studies in adolescence and the early adult years and, in all series, a predominance of records on women. However, except for the oldest age group, the samples are drawn from all sections of the country. Data from the far west predominate in the oldest age groups.

Figures 1 and 2 express the mean and the range of intakes of calcium. The data for children are used on both figures. There is some overlapping of age groups in that there are a few 16-year-olds in the 13–15 group and some individuals listed as 24–49 years may have been older. The numbers involved in each case are small and it is doubtful that the trends of intake are greatly influenced by the inclusion of the few studies which could not be fitted exactly into the classification.

Each dot represents the mean intake from one study. Studies of non-white groups and of pregnant women were tabulated separately but, since the means and ranges were within the spread of data of the respective age and sex groups, they have not been separated in the figures. Ranges were plotted in absolute values when recorded by the author; other studies are reported as the mean, plus or minus three standard deviations. It was soon apparent that the distribution of reported calcium intakes did not follow the classical bell shaped curve since three standard deviations less than the mean resulted in negative values. Since the data are drawn from healthy populations, zero intakes of calcium are an absurdity and it is presumed that the figures are biased by a skewing of the distributions in the direction of very high intakes. Thus zero intakes of calcium as graphed express the least intake of calcium compatible with an amount of food to support health and activity. From the few actual ranges reported, about 150 mg per thousand calories of food is an amount which might be termed the lowest practical intake of calcium in our dietary patterns.

A gradual increase in calcium intake occurs through the age group 16–24 years. During the first 12 years, mean intakes tend to average slightly less than the Recommended Allowances of the Food and Nutrition Board (10) for calcium in all groups. Mean intakes of adolescent girls

TABLE 1. NUMBER OF INDIVIDUALS REPRESENTED IN SURVEY STUDIES OF CALCIUM INTAKE

Age Group, yr.	Number of Individuals		
	Male	Children	Female
1– 3		317	
4– 6		406	
7– 9		2430	
10–12		752	
13–15	1278		1555
16–24	2349		2741
25–49	1076		3836
50–69	233		1350
More than 70	142		233
Total	5078	3905	9815

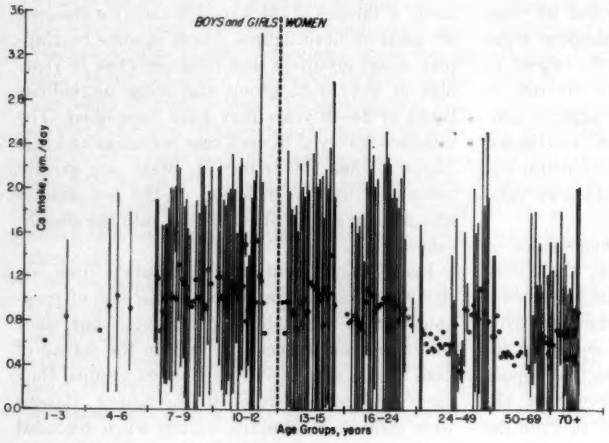


FIG. 1. Mean and range of intake of calcium of children and women. Studies of the literature 1942–1958. Each dot represents the mean of one study. The vertical line is the actual range or the mean plus or minus 3 standard deviations, depending on which statistic was reported.

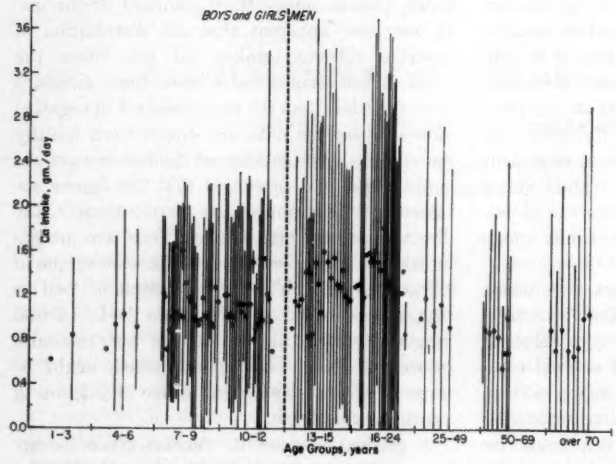


FIG. 2. Mean and range of intake of calcium of children and men. See figure 1 for description of symbols.

are slightly less than the Allowances and the intakes of women throughout the span of years represented by these studies are about 200 mg less than the Allowances. Adolescent boys also average slightly less than recommended allowances.

At all ages after 12, men consume more calcium than women and, at all ages, there are individual men and women who ingest two to four times the Recommended Allowances of calcium; there also are at least an equal number of individuals in all groups who take the least amount of calcium consistent with eating adequate calories. Many adults, especially women, ingest very little calcium.

At least three large clinics (52–54) have found osteoporosis the most common bone disease in middle age and, contrary to earlier publications, report that dietary histories suggest a life time of poor food intakes including limited calcium and protein as well as other nutrients. Eighty per cent of the osteoporosis of middle age occurs in women (55). While the use of estrogens provides for an immediate stimulus for osteoblastic activity, Larson (53) reports that significant recalcification with prolonged clinical remission is obtained only with a general improvement of the dietary, including an increase in calcium intake. Pearson (56) has reported marked recalcification of affected bone following removal of the ovaries in patients with breast cancer and bony metastases with no treatment other than a good diet.

No evaluation of the calcium intakes presented can be made without consideration of some of the factors which influence the utilization of this mineral. That healthy people are able to adapt to a wide range of intake of nutrients is generally accepted. In the controlled balance experiment, adaptation is commonly measured under conditions planned to provide constant and adequate amounts of all dietary requirements except the nutrient in question. Meal periods are prescribed, menus simplified for the convenience of the laboratory, and consumption of all food offered is expected. Subjects who cannot accept this type of discipline are not used, nor are periods of ill health, holidays or other distractions included. Activity and rest usually are controlled.

Balance studies of people in technically underdeveloped countries, on their native diets, show a marked capacity to adapt to low calcium intakes, but limitation of food has forced adaptation over many generations under conditions which, in some respects, are not unrelated to the controlled balance experiment. Asiatic children seem to adapt well to the low diets. Yet the simple addition of calcium lactate to the diet of preschool and school age Indian children resulted in a prompt increase in rate of linear growth (57, 58). Greulich's (59) studies of the growth of American children of Japanese parentage compared to that of their Tokyo counterparts show the same rapid adaptation to increased amounts of many nutrients. We know of no studies of adaptation of European or of American children to very low calcium diets.

There are a number of studies on record of the adaptation of adults to reduced intakes of calcium. The most complete of these is the distinguished work of Malm (60) using male inmates of a Norwegian prison. The diet fed in this case was characteristic of the population of men studied but, even here, prison surroundings served to limit variety of food and to routinize meals.

The adaptations which an individual makes for himself in this country are to a ration of mixed foods. He is limited only by his buying power and capacity to digest and enjoy food. Moreover, he eats when and where it is convenient. Figure 3 shows two distributions of intakes and retentions of calcium adapted from the literature. The upper figure (61–89) includes all of the available studies on young women in this country in which data were collected under conditions in which the amount or source of calcium was the principal

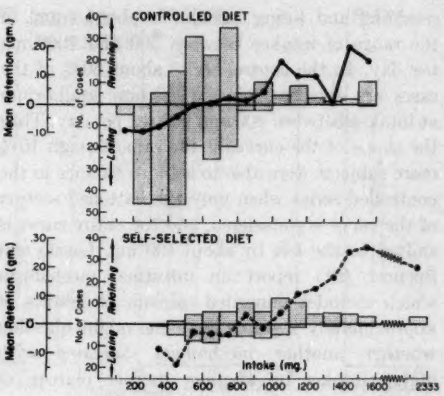


FIG. 3. Mean retention of young adult women and number of cases retaining or losing calcium on intakes from 200 to 1600 mg per day.

experimental variant. The irregular line represents the mean retention at any given intake. All studies included a preliminary period on less than 400 mg calcium per day. The lower curve is a similar expression of the data reported by McKay *et al.* (90) in which the subjects ate freely from the food offered in college communities but weighed and sampled all food and submitted to the collection of excreta for two 5-day periods of study. It is obvious that intake and retention are related in both instances. From the slope of the regression lines, the subjects of the controlled studies achieved a mean equilibrium at intakes of 650 mg per day as against 820 mg for the self-selected diet series. That this may be an unduly simple expression of the data is suggested by the blocks in which the number of cases losing or retaining calcium is recorded about the line of zero retention. In both sets of data lowest intakes resulted in strongly negative retentions. When more than a gram of calcium is ingested, there is a trend toward an increased retention. Between intakes of 400 and 1000 mg per day, mean retentions vary within the range of experimental error. In other words, the regression of calcium retention on intake could be more accurately described as an S curve than as a straight line.

Intakes of from 400 to 1000 mg calcium per day are of special interest since 400 mg is about the least amount anyone eating from our food supplies is apt to ingest from a diet adequate in other respects but lacking milk. About 1000 mg calcium per day are found in a well balanced adult diet containing a pint of milk. In the group with self-selected diets, the number of cases

retaining and losing calcium is about equal in the range of intakes between 500 and 1000 mg per day. In the control series about 60% of the cases are able to maintain calcium equilibrium at intakes between 400 and 900 mg per day. Thus the shape of the curves is the same though 10% more subjects were able to achieve balance in the controlled series when only the flattened section of the curve is considered, and the entire curve is shifted to the left by about 100 mg. Laszlo and Spencer (91) report an intestinal mechanism which excludes unneeded calcium at intakes of approximately 1 gm or less. One might question whether another mechanism operates with higher intakes or whether another plateau of equilibrium is reached before storage decreases.

A similar distribution was found in every series of data on adults or children separated in this way. It is suggested that one effect of imposing a controlled diet is to separate more clearly the individuals who adapt readily to a low calcium intake from those who adapt with difficulty or not at all.

Malm (60) recorded results in keeping with this hypothesis. Thirty-nine adult male subjects were fed 900 to 1000 mg of calcium for periods from 70 to 532 consecutive days. About half of the group neither gained nor lost calcium significantly. The remaining half showed a cumulative gain of 20 gm of calcium or more during the study. Retention was still high for some subjects after 8 and 9 months of feeding but in those subjects who adapted before the end of the period, the preliminary retentions were followed by weeks of equilibrium. Twenty-seven of the group were then given lower calcium intakes varying from 397 to 647 mg per day. Of this group, 40% were able to adapt either immediately or eventually without significant over-all loss of body calcium. The other 60% either adapted without replacement of calcium lost, or (3 subjects) were unable to adapt to the lowered intakes. About a fourth of the subjects who adapted with difficulty lost 20 gm or more of calcium before equilibrium was obtained.

A fourth of the subjects studied by Malm were thought to have some x-ray evidence of decalcification of the skeleton. Since 35 to 50% of skeletal mineral must be lost before the x-ray shows decalcification in adult bones, it could be assumed that a good many other subjects who continued to store calcium on 900 to 1000 mg per day may have had lesser degrees of demineralization. There is no suggestion, however, that adult males continue to store calcium indefinitely, within these limits of intake. Many individual protocols suggest that the man reduced to 650 mg of calcium or less per day adjusts at the cost of a lowered concentration of mineral in the skeleton. If this is true under the relatively protected prison situation, the losses of adaptation would be expected to be exaggerated under conditions of free living. Gross calcium losses of 20 gm or more in less than a year cannot be considered negligible.

In studies of children an allowance must be made for storage of calcium to support growth. Figure 4 gives the mean intake and range of intakes for studies on children 10–17 years as derived from the literature (92–100) and certain unpublished data from the State University of Iowa. Three intake ranges are compared from a low of 0.7 to 1.5 gm per day to a high of 2 to 2.4 gm per day. Maximum retentions were approximately the same for the three intakes. The values show that efficiency in the utilization of calcium varies widely in any group of children. Although a gradual increase in the mean level of retention is apparent, increases of intake beyond a given amount have little effect on the retention of this element, as the highest intake produced an increase in mean retention much less than that measured between the low and median intakes. For those children with the least efficiency in utilization, an increase in intake, when a well balanced diet is offered, serves to increase retention and a minimum intake cannot be expected to permit normal calcification in the majority of children.

Additional evidence in support of this point is shown in figure 5. This figure gives the retentions of two groups of adolescent children. The boys and girls represented by the lower curves are children who were known to have received a borderline diet in many nutrients previous to admission to the ward. The diet did, however, include a pint to a pint and a half of milk daily and was adequate in calories. The upper curve gives the retentions of a similar group of children from an orphanage providing a nutritionally excellent diet. Preliminary adaptation periods were the same in both studies as was the amount of food and vitamin D consumed. Six months on a good diet were required before the children known to have had a borderline diet were able to equal the retentions of previously well fed children, but the children coming from the poorer environment were able to take advantage of

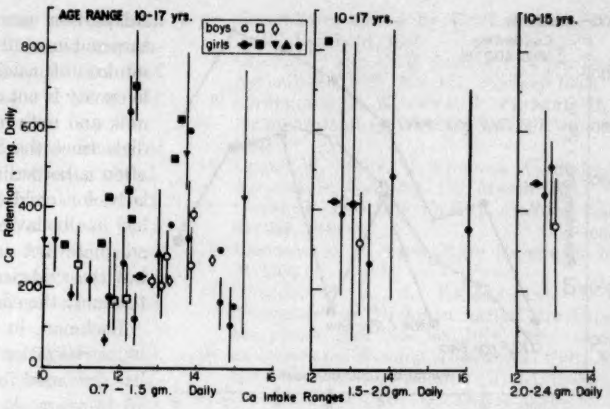


FIG. 4. Mean retention and range of retention of children 10–17 years old, with three ranges of calcium intake. *1)* Symbols: ○ ● State University of Iowa studies of individual male and female children; –●– McKay, Smith and Smith (95); –■– Wang, Kaucher and Wing (99); ■ Sherman and Hawley (97); □ Macy (98); ▼ Johnston, Schlaphoff and McMillan (92); ▲ Johnston (93). *2)* Vertical lines: range of retention. *3)* Small symbols: studies on one child over a period of time; large symbols: group studies.

increasing intakes of both calcium and vitamin D.

Figure 6 depicts the mean retention of a large series of Iowa children from infancy to 12 years of age. The children were given graded intakes of calcium in a series of studies covering several months. Vitamin D, in the amount of 300–400 units daily was then added to the regimen, and the series repeated. The data have been compared to a 'theoretical requirement' calculated from average rate of growth, percentage of body weight due to skeleton and percentage of calcium in mammalian bone when the animal has lived on an ample diet (Curve R).

The data show that vitamin D was essential to allow adequate mean calcium retention for the children when milk intake was restricted to 1.5 pints per day or less. The mean retentions with 1.5 pints of milk and added D almost duplicated curve R, so are not shown on the slide. One quart of milk and 400 units D permitted retention of calcium well above the theoretical requirement. Data are available for an extension of the theoretical growth curve into adolescence. A mean retention of 400 mg per day is necessary for several years if the adolescent girl is to have a fully calcified skeleton by 18 years. Since many girls from all stations of life marry and bear children before 20, early mineralization is important for the girl. Retentions of 400 mg per day were found only with intakes of calcium in the range of 1.5–1.6 gm per day when 400 units of vitamin D were used.

In interpreting these findings, we emphasize the well known fact that all such metabolic studies are carried out under as nearly optimum conditions as possible. Performance is then

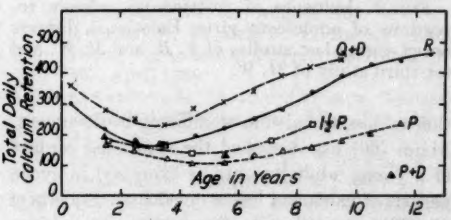


FIG. 5. Mean calcium retentions of children 0–12 years on graded intakes of milk with and without 350 units vitamin D. Symbols: P—1 pint milk per day; Q—1 quart milk per day; D—added vitamin D; R—theoretical retention curve. Unpublished studies from the State University of Iowa.

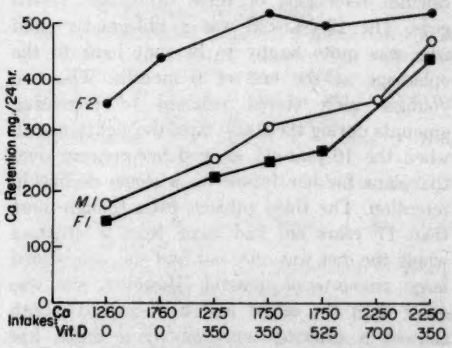


FIG. 6. Calcium retentions of children with previously good diet (upper curve) and poorer diet (lower curves) on gradually increasing intakes of calcium and vitamin D. Each period represented a month of study.

close to the child's maximum and cannot be considered representative of his mean annual retention.

The role which emotions play in the conserva-

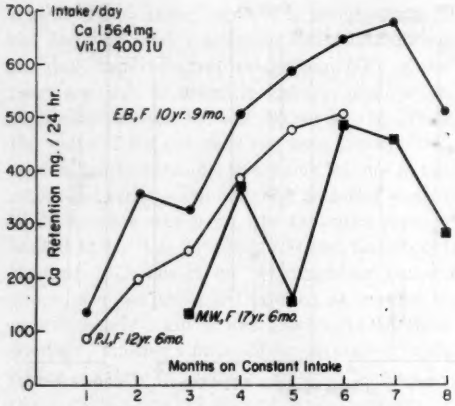


FIG. 7. Influence of emotion on calcium retentions of adolescent girls. Emotional distress noted during last studies of *E. B.* and *M. W.* and for third study of *M. W.*

tion or loss of calcium is difficult to document. Malm (60) has described the emotional conflict of a young adult prisoner as expressed in cyclic periods of gains and losses of calcium. Roberts *et al.* (38) reported wide fluctuations in calcium conservation in a healthy woman in her sixties who had two sons in active service during World War II. Both of these subjects were able to recoup losses sustained during periods of tension when the tensions were released. Figure 7 shows the calcium retentions of three orphanage reared girls. The 12-year-old was a phlegmatic child who was quite happy to be sent back to the ophanage at the end of 6 months. The two younger girls stored calcium in increasing amounts during the study until the eighth month when the 10-year-old showed her concern over the plans for her future by a sharp decline in retention. The third subject, even though more than 17 years old had come from a situation where the diet was only fair and she, too, stored large amounts of mineral. However, she was older than the others and in the third month showed a growing boredom by a much less favorable storage. Arrangements were made to make the situation more interesting and she again stored calcium until the final period when she was told of the plans for her future.

DISCUSSION

Many diverse pieces of experimental evidence have been presented. Many individuals exist for a lifetime on a minimum amount of calcium. Adaptation occurs during growth in bone structure and in adult size. How successfully minimum intakes of calcium promote adult vigor and longevity is not clear. Our society is one in which milk and milk products have had a place in our diets since the Stone Age. A diet without milk often is borderline in supply of protein, particularly for children and women, and is usually low in riboflavin. This is not to suggest that an adequate diet cannot be obtained without milk but the evidence from our population groups is that only the educated few actually do so.

Backman, in 1948 (101), reported that with industrialization in Europe, the age of menarche has decreased from an average of 16–17 years to 13–14 years. At the same time, the menopause, which occurred on the average at age 45 during the 15th century to 1830, now tends to be delayed until 48. This measures an increase of 6–8 years in the productive life cycle of women, most of the increase having occurred during the last 100 years. While it is recognized that more than food intake is involved, increased buying power means a better diet for the masses of people. It is of some interest that, of all the longevity studies on animals, only those of Sherman on the white rat increased length of life by an extension of the active adult portion of the life span (102).

A moderate consumption of milk by humans eating adequate calories serves to bring protein, calcium, riboflavin and vitamin A to intakes approaching the Recommended Allowances and without milk, only those individuals who deviate sharply from our usual dietary patterns tend to ingest calcium and riboflavin in equal proportions. Few young children or adolescent girls will consume enough meat or fish to make up for the lack of milk in supplying protein.

The food intake studies presented again demonstrate that adult women and adolescent children are the least successful in obtaining calcium from the family table and the average adult man gets more than his share. Since much of the calcium is derived from milk at the intakes recorded, he also gets more than his share of the protein, riboflavin and, perhaps, the vitamin A.

We may question whether the child who stores little calcium does so because of any great economy in utilization or because his whole body is less well nourished and a certain maturity of all tissues is necessary for efficient utilization. Silberberg and Silberberg (103) point out that while restriction in food may be indicated in the adult, higher mammals are less adaptable to drastic

reduction in food supply during growth than lower forms of animals. In the human, preparation for pregnancy as well as the condition itself may be an important determinant of the ability of the infant to thrive.

In our society, a child has a wide choice of foods in his own environment and may not always choose an adequate daily diet. In comparison with the changes in retention caused by a diet inadequate in other factors, by fatigue and emotion, any errors in the metabolic experiment due to manipulation, analytical procedures and the like, are relatively minor.

In the daily home life, one would expect children to show a lower mean retention of calcium with a given intake than those reported in the studies quoted in this paper. The 'osteoporosis of adolescence' noted by Todd (104) in a majority of urban children during the thirties is evidence of a nutritional deficiency of considerable magnitude, for diagnosis was made by roentgenogram. There is no evidence that vitamin D replaces the need for calcium during growth or, so far as can be judged by experimental evidence, in the adult.

No attempt has been made in this paper to consider the recent work with radioactive calcium although the importance of the papers is realized. It may be remarked in passing, that the data obtained to date are in keeping with those of the controlled balance experiment without the isotopically labeled ion. Rather, an attempt has been made to present the way in which our calcium supplies are distributed among our population and to point up some of the over-all factors in calcium exchange which will influence the individual's ability to adapt when living in the conditions of our society rather than in the metabolic ward.

There is a very practical aspect to the provision of nutrients from the grocery store. The real point at issue is not whether the total milk supplies of our population are excessive for good health but whether they are reaching the individuals in the family who need them most.

REFERENCES

1. Lusk, G. *The Elements of the Science of Nutrition*. Philadelphia: Saunders, 1928.
1a. Ohlson, M. A., L. Jackson, J. Boek, D. C. Cederquist, W. B. Brewer and E. G. Brown. *Am. J. Pub. Health* 40: 1101, 1950.
2. Montgomery, E. R. and C. H. Kardell. U. S. Dept. Commerce, Domestic Series, Bull. No. 38.
3. Webb, B. H. In: *Handbook of Food and Agriculture*, edited by F. C. Blanck. New York: Reinhold, 1955.
4. Clark, F. and C. B. LeBovit. U. S. Dept. Agriculture, Bull. No. 127, January 1955.
5. Stiebeling, H. K. and E. F. Phipard. U. S. Dept. Agriculture Circular No. 507, January 1939.
6. Clark, F. and L. J. Fincher. U. S. Dept. Agriculture, Bull. No. 112, March 1954.
7. Clark, F. and J. B. McKay. *J. Home Econ.* 38: 333, 1946.
8. Phipard, E. F. Agriculture Handbook No. 56, 1952.
9. Ohlson, M. A., L. Kelley and G. G. Quackenbush. Michigan Agriculture Exper. Sta., Tech. Bull. No. 258, July, 1956.
10. *Recommended Dietary Allowances*. Pub. No. 589, Nat. Research Council (U. S.), 1958.
11. Dean, W. T., B. C. Davis and S. L. McConnell. Virginia Polytechnic Institute, Tech. Bull. No. 122, August 1954.
12. Tucker, R. E. and P. T. Brown. Rhode Island Agricultural Exper. Sta., Bull. No. 327, April 1955.
13. Leverton, R. M. and J. Pazur. Nebraska Agricultural Exper. Sta., Misc. Pub. No. 5, April 1957.
14. Eppright, E. S., V. D. Sidwell, P. P. Swanson. *J. Nutrition* 54: 371, 1954.
15. Young, C. M., B. M. Einset, E. L. Empey, V. U. Serraon. *J. Am. Dietet. A.* 33: 374, 1957.
16. Leichsenring, J. M., E. G. Donelson, H. H. Deinard, M. S. Pittman, M. Cooprider and V. Haggart. *J. Home Econ.* 35: 583, 1945.
17. Leverton, R. M., J. M. Ellison, M. T. Childs, A. F. Carver and D. Twardock. *J. Home Econ.* 51: 33, 1959.
19. North Central Regional Publication No. 59; Iowa Agricultural Exper. Sta.; Research Bull. No. 434, December 1955.
20. Jeans, P. C., M. B. Smith and G. Stearns. *J. Am. Dietet. A.* 31; 576, 1955.
21. Hoover, C. A. and M. C. Coggs. *J. Home Econ.* 40: 193, 1948.
22. Lantz, E. M. and P. Wood. *J. Am. Dietet. A.* 34: 138, 1958.
23. Lantz, E. M. and P. Wood. *J. Am. Dietet. A.* 34: 1199, 1958.
24. Warnick, K. P., S. V. Bring and E. Woods. *J. Am. Dietet. A.* 31: 486, 1955.
25. Odlund, L. M., L. Page and L. P. Guild. *J. Am. Dietet. A.* 31: 1134, 1955.
26. Fisher, K. A. and M. L. Dodds. *J. Am. Dietet. A.* 34: 392, 1958.
27. Leverton, R. M. and J. Ellison. *J. Home Econ.* 45: 326, 1953.
28. Clark, F. and L. J. Fincher. U. S. Dept. Agriculture, Bull. No. 112, March 1954.
29. McMillan, T. J. and R. M. Leverton. *J. Home Econ.* 35: 514, 1943.
30. Leverton, R. M. and T. J. McMillan. *J. Home Econ.* 36: 225, 1944.

31. Peel, R. M. and M. L. Dodds. *J. Am. Dietet. A.* 33: 1150, 1957.
32. Tucker, R. E., C. L. Brine and M. S. Wallace. *J. Am. Dietet. A.* 34: 819, 1958.
33. Wilcox, E. B. and L. S. Galloway. *J. Am. Dietet. A.* 30: 345, 1954.
34. McGanity, W. J., E. B. Bridgforth, M. P. Martin, J. A. Newbill and W. J. Darby. *J. Am. Dietet. A.* 31: 582, 1955.
35. Murphy, G. H. and A. W. Wertz. *J. Am. Dietet. A.* 30: 34, 1954.
36. Kelley, L., M. A. Ohlson and L. J. Harper. *J. Am. Dietet. A.* 33: 466, 1957.
37. Ohlson, M. A., P. H. Roberts, S. A. Joseph and P. M. Nelson. *J. Am. Dietet. A.* 24: 286, 1948.
38 Roberts, P. H., C. H. Kerr and M. A. Ohlson. *J. Am. Dietet. A.* 24: 292, 1948.
39. Manning, I. H. and D. F. Milam. *South. M. J.* 36: 373, 1943.
40. Cooperative Nutritional Status Studies in the Western Region. Utah Exper. Sta. Bull. No. 383.
41. Winters, J. C. and R. E. Leslie. *J. Nutrition* 26: 443, 1943.
42. Winters, J. C. and R. E. Leslie. *J. Nutrition* 27: 185, 1944.
43. Hayes, O. B., L. J. Bowser, M. F. Trulson. *J. Gerontol.* 11: 154, 1956.
44. Lyons, J. S. and M. F. Trulson. *J. Gerontol.* 11: 66, 1956.
45. Beal, V. A. *J. Nutrition* 53: 499, 1954.
46. Greenwood, M. L. and B. N. Lonsinger. *J. Am. Dietet. A.* 20: 671, 1944.
47. Johnston, F. A. *J. Am. Dietet. A.* 19: 416, 1943.
48. Young, C. M. *J. Am. Dietet. A.* 22: 25, 1946.
49. Wakefield, L. and M. Potgieter, *J. Am. Hosp. Assn.* Nov. 1, 1957.
50. Northeast Regional Publication No. 11: Rhode Island Agricultural Exper. Sta., Bull. No. 319, 1952.
51. Wiehl, D. G. and K. Berry. *Milbank Mem. Fund Quart.* 23, 353: 1945.
52. Smith, R. W., Jr. *Family Circle, March,* 1959.
53. Larson, C. B. Personal communication.
54. Whedon, G. D. *Fed. Proc.* 18: 1112, 1959.
55. Hall, R. A. *Harper Hosp. Bull.* 16: 259, 1958.
56. Pearson, O. H. In: *Hormones and the Aging Process,* edited by E. Pincus, New York: Acad. Press, 1956, p. 147.
57. Aykroyd, W. R. and B. G. Krishnan. *Lancet* 153: 1938.
58. Aykroyd, W. R. and B. G. Krishnan. *Indian J. M. Res.* 27: 409, 1939.
59. Greulich, W. W. *Science* 127: 515, 1958.
60. Malm, O. J. *Calcium Requirement and Adaptation in Adult Men.* Oslo: Oslo University Press, 1958.
61. McKay, H., M. B. Patton, M. S. Pittman, G. Stearns and N. Edelblute. *J. Nutrition* 26: 153, 1943.
62. Breiter, H., R. Mills, E. Rutherford, W. Armstrong and J. Outhouse. *J. Nutrition* 23: 1, 1942.
63. Breiter, H., R. Mills, J. Dwight, B. McKey, W. Armstrong and J. Outhouse. *J. Nutrition* 21: 351, 1941.
64. Bricker, M. L., J. M. Smith, T. S. Hamilton and H. H. Mitchell. *J. Nutrition* 39: 445, 1949.
65. Patton, M. B. and T. S. Sutton. *J. Nutrition* 48: 443, 1952.
66. Leichsenring, J. M., L. M. Norris, S. A. Lamison, E. D. Wilson and M. B. Patton. J. *Nutrition* 45: 407, 1951.
67. Patton, M. B. *J. Nutrition* 55: 519, 1955.
68. Schofield, F. A., D. E. Williams, E. Morrel, B. B. McDonald, E. Brown and F. L. MacLeod. *J. Nutrition* 59: 561, 1956.
69. Patton, M. B., E. D. Wilson, J. M. Leichsenring, L. M. Morris and C. M. Dienhart. *J. Nutrition* 50: 373, 1953.
70. Mallon, M. G., L. M. Johnson and C. R. Darby. *J. Nutrition* 6: 303, 1933.
71. Mallon, M. G., L. M. Johnson and C. R. Darby. *J. Nutrition* 5: 121, 1932.
72. Mallon, M. G., L. M. Johnson and C. R. Darby, *J. Nutrition* 6: 303, 1933.
73. Kramer, M. M., M. T. Potter and I. Gillum. *J. Nutrition* 4: 105, 1931.
74. McLaughlin, L. *J. Biol. Chem.* 74: 455, 1927.
75. Blatherwick, N. R. and M. L. Long. *J. Biol. Chem.* 52: 125, 1922.
76. Mallon, M. G., R. Jordan and M. Johnson. *J. Biol. Chem.* 88: 163, 1930.
77. Fincke, M. L. and E. A. Garrison. *Food Res.* 3: 575, 1938.
78. Milles, J. and J. B. Reed. *Biochem. J.* 41: 273, 1947.
79. Kunerth, B. L. and M. S. Pittman. *J. Nutrition* 17: 161, 1939.
80. Pittman, M. S. and B. L. Kunerth. *J. Nutrition* 17: 175, 1939.
81. Burton, H. B. *J. Biol. Chem.* 85: 405, 1930.
82. Pittman, M. S. *J. Nutrition* 5: 277, 1932.
83. Rose, M. S. *J. Biol. Chem.* 41: 349, 1920.
84. Rose, M. S. and G. MacLeod. *J. Biol. Chem.* 57: 305, 1923.
85. Sherman, H. C. and L. H. Gillett and H. M. Pope. *J. Biol. Chem.* 34: 373, 1918.
86. Sherman, H. C. and J. C. Winters. *J. Biol. Chem.* 35: 301, 1918.
87. Sherman, H. C., J. C. Winters and V. Phillips. *J. Biol. Chem.* 39: 53, 1919.
88. Sherman, H. C., L. Wheeler and A. B. Yates. *J. Biol. Chem.* 34: 383, 1918.
89. Kramer, M. M., E. Latzke and M. M. Shaw. *J. Biol. Chem.* 79: 283, 1928.
90. McKay, H., M. B. Patton, M. A. Ohlson M. S. Pittman, R. M. Leverton, A. G. Marsh, G. Stearns and G. Cox. *J. Nutrition* 24: 367, 1942.
91. Laszlo, D. and H. Spencer. In: *Hormones and the Aging Process.* New York: Acad. Press, 1956, p. 175.
92. Johnston, F. A., D. Schlaphoff and T. McMillan. *J. Nutrition* 41: 137, 1950.
93. Johnston, J. A. *Am. J. Dis. Child.* 59: 287, 1940.

94. JOHNSTON, J. A. *Am. J. Dis. Child.* 62: 708, 1941.
95. MCKAY, B., E. SMITH AND J. M. SMITH. *Fed. Proc.* 7: 293, 1948.
96. HERBST, O. *Ztschr. Kinderh.* 7: 161, 1913.
97. SHERMAN, H. C. AND E. HAWLEY. *J. Biol. Chem.* 53: 375, 1922.
98. MACY, I. G. *Nutrition and Chemical Growth in Childhood.* Vol. II. Springfield; Thomas, 1946.
99. WANG, C. C., M. KAUCHER AND M. WING. *Am. J. Dis. Child.* 51: 801, 1936.
100. WANG, C. C., M. KAUCHER AND M. WING. *Am. J. Dis. Child.* 52: 41, 1936.
101. BACKMAN, VON G. *Acta anat.* 4: 421, 1948.
102. SHERMAN, H. C. *Chemistry of Food and Nutrition.* New York: Macmillan, 1952.
103. SILBERBERG, M. AND R. SILBERBERG. *Physiol. Rev.* 35: 347, 1955.
104. TODD, T. W. *J. Pediat.* 3: 415, 1933.

FACTORS INFLUENCING CALCIUM ABSORPTION[1]

HAROLD E. HARRISON

Pediatric Division, Baltimore City Hospitals and the Pediatric Service, Johns Hopkins University School of Medicine, Baltimore, Maryland

DIVALENT IONS are as a group absorbed much less readily and less completely from the intestinal lumen than are most univalent ions. Because of the importance of calcium as an essential nutrient this failure of complete absorption is often regarded as a lack of absorptive efficiency or a disadvantageous characteristic of intestinal function. Not only is dietary calcium incompletely absorbed but if the dietary intake is reduced to minimal amounts fecal calcium loss exceeds the intake. This so-called endogenous calcium loss is believed to represent calcium of intestinal secretions which fails of complete reabsorption from the intestine and thus is wasted in the excretions.

Incomplete absorption of the calcium ingested can not, however, be regarded simply as lack of efficiency of intestinal function since intestinal absorption of calcium can have a homeostatic function. This is not true of such cations as sodium or potassium. These ions are fairly completely absorbed from the intestine in the absence of gross disturbances of intestinal function and the homeostatic regulatory control is their renal excretion. In the instance of calcium, renal excretion can not be the sole regulatory mechanism. Almost all of the filtered calcium load is reabsorbed by the tubules and this reabsorption can be essentially complete as in patients with osteomalacia or nutritional calcium deficiency. Somewhat less complete reabsorption is found with high calcium intakes or acidosis but the range of calcium excretion in the urine is limited, and any large increase in urine calcium can result in precipitation of insoluble calcium salts leading to renal calcinosis or renal calculi. If diets high in calcium are ingested hypercalciuria is prevented either by rapid uptake of absorbed calcium by the skeleton, or by rejection of calcium by the intestine so that the amount of calcium absorbed is in the range which can be excreted in the urine without precipitation. The first mechanism can operate in the growing animal but in the adult animal the second mechanism is presumably the important one unless there has been prior skeletal demineralization.

Before developing the possible homeostatic role of the intestinal mucosa in controlling or regulating absorption of calcium from the lumen, I shall discuss those factors which are necessary for calcium absorption to take place. Calcium is apparently absorbed in the ionic form and intestinal uptake is diminished by agents which reduce the concentration of ionic calcium in the intestinal contents. A variety of anions which precipitate, or complex ionic calcium such as oxalate, phytate, phosphate, or possibly sulfate, may interfere with calcium absorption if present in excess (1). The effect of pH of the intestinal contents on absorption is probably also related to the concentration of calcium ion in solution. There is much conflicting data as to the extent to which phosphate, phytate or alkali intake may actually reduce absorption of calcium. Part of the contradiction may be due to the operation of other factors which increase efficiency of intestinal absorption of

[1]The author's investigations cited in this paper were aided by Grant A-668 from the National Institutes of Health.

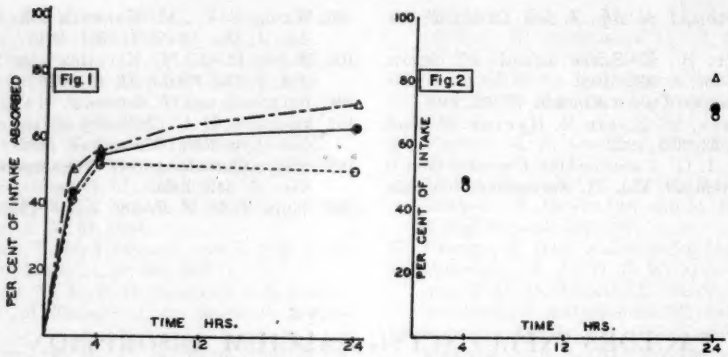


FIG. 1. Effect of vitamin D on absorption of Ca^{45}. Ten mg of Ca as $CaCl_2$ given by stomach tube to non-fasted rats fed high calcium, low phosphorus diet. ○—vitamin D deficient; ●—vitamin D 100 U once weekly for 3 weeks; △—vitamin D 3000 U 72 hr. before the start of the experiment. The percentage of the administered Ca^{45} absorbed from the intestine at the designated time is shown by the curves.

FIG. 2. Effect of vitamin D on absorption of Ca^{45} in animals previously deprived of calcium. 10 mg of Ca as $CaCl_2$ given by stomach tube to rachitic rats fed low calcium diet for 3 days and then fasted for 24 hr before experiment to remove calcium from intestinal tract. Symbols as in fig. 1. The percentage of the administered Ca^{45} absorbed at 4 and 24 hr after intragastric administration is shown by the points.

calcium at low concentrations of calcium ion in the intestinal contents. The most significant of these is vitamin D. I need not discuss the large body of evidence which shows that vitamin D is necessary for efficient intestinal absorption of dietary calcium, but shall confine myself to a few studies which may provide some clues to the possible mechanisms of this effect.

Absorption of calcium can be studied in rats by stomach tube administration of a calcium load tagged with Ca^{45}, and determination of the rate of removal of the calcium from the intestine (2). It is found that there is an initial very rapid rate of absorption both in vitamin D deficient and vitamin D supplemented rats. A slight effect of vitamin D is probably present at this phase. After the first few hours absorption ceases in the vitamin D depleted animals but continues in the vitamin D treated rats so that the total overall absorption is significantly greater in the vitamin D treated animals. This effect is shown in our own experiments in which the calcium was given as the calcium chloride (fig. 1). A somewhat different curve of absorption is seen in experiments of Lindquist (3) in which a larger load of calcium as calcium lactate solution, was used; but in these experiments also the initial very rapid phase of absorption is found, with leveling off of absorption in the vitamin D depleted animals at a lower final percentage of fed calcium than in vitamin D supplemented animals. One possible explanation for the differences in the absorption curves between vitamin D deficient and vitamin D supplemented rats may be the relation of Ca ion concentration to absorption. In the initial phase of absorption in the proximal gut the concentrations of Ca ion in intestinal contents are maximal. Under these conditions rapid absorption occurs from the rat intestine even in the absence of vitamin D. As the test material moves down the intestine the fluid becomes more alkaline and the test salt is mixed with unabsorbed intestinal contents including previously ingested food residue. It is likely that the Ca ion concentration in the fluid is diminished, and under these conditions vitamin D is needed for absorption. In other words the transport mechanism may be vitamin D dependent at low concentrations of Ca ion in the fluid bathing the mucosa but less so at high concentrations of calcium. In support of such a postulation are the findings of an experiment in which Ca^{45} labeled $CaCl_2$ was given to rats who had been given a low calcium diet for a period preceding the experiment. The intestinal contents were thus freed of dietary calcium and all of the calcium in the lumen was in the form of the soluble $CaCl_2$ salt. Under these conditions a higher proportion of the calcium given was absorbed than in the fed animals and no real difference between vitamin D deficient and supplemented rats was found (fig. 2). Another experiment trying to demonstrate the same point is shown in figure 3. In the previous studies 10 mg of total Ca had been given whereas in this experi-

ment only 150 μg of labeled Ca was given. Almost all of the administered calcium was absorbed by the vitamin D supplemented rats during the 4-hour period whereas absorption was less uniform in the vitamin D deficient rats and ranged from 47 to 95% of the test dose. This again suggests that vitamin D increases the efficiency of absorption of calcium particularly at low concentrations of available calcium. This effect may explain the variability of results obtained in experiments attempting to show the effect of phytate or of phosphate on uptake of dietary calcium. Although in some experiments these complexing agents may reduce calcium retention, on others little effect is found. The action of vitamin D in permitting efficient absorption of calcium at low concentrations of calcium may counteract the effect on calcium absorption of factors which reduce calcium ion concentration in the intestinal contents.

The mechanism whereby vitamin D effects this increased efficiency of calcium transport by intestinal mucosa is not known. One of the theories that has some experimental support is that based upon the citrate increasing effect of vitamin D. A number of investigators have found that one of the consistent effects of vitamin D is an increase in serum citrate levels and bone citrate concentrations (4-6). Increases of citrate concentration in other tissues such as intestine and kidney (5) have been reported but these may be the result mainly of increased extracellular citrate concentrations in the first instance and increased urine citrate levels in the second. DeLuca and his colleagues (7, 8) however, have reported that vitamin D reduces the utilization of citrate by rat kidney slices, and mitochondrial suspensions, suggesting that this may be the basis for increased citrate accumulation following vitamin D. Our own analyses of tissues have indicated that intracellular citrate is not increased in heart and intestine following vitamin D but that there may be a change in the equilibrium between intracellular and extracellular citrate possibly due to increased efflux of citrate into extracellular fluid under the influence of vitamin D. The role of citrate in intestinal absorption of calcium is of interest since the intestine like the musculoskeletal system adds citrate to the blood perfusing it (9) (fig. 4). Following an intraintestinal calcium load the serum citrate rises along with the serum calcium and the arterial-portal venous difference of citrate remains positive and is perhaps increased (fig. 5 and 6). No

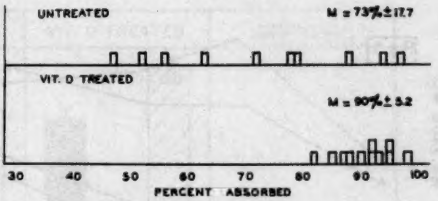


FIG. 3. Effect of vitamin D on absorption of Ca^{45}. 0.15 mg of Ca given as $CaCl_2$ to untreated rachitic rats and to rachitic rats given 3000 U vitamin D 72 hr. before experiment. Each square indicates the result in an individual rat in terms of percent of the administred Ca^{45} absorbed at 4 hr. after the intragastric administration.

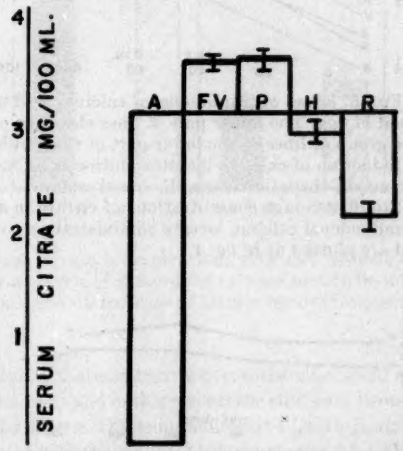


FIG. 4. Citrate concentrations in arterial serum and arterio-venous differences of citrate in serum obtained from femoral, portal, hepatic and renal venous blood of dog. The height of the column labeled A indicates concentration of citrate in arterial serum. The arterio-venous differences are represented by the columns plotted from the horizontal line representing the arterial concentration. Those above the line indicate addition of citrate to the perfusing blood whereas those below the line indicate removal of citrate.

calculations of the absolute increments of calcium and of citrate in the venous blood draining the intestine per unit of time can be made in the absence of blood flow measurements. The increases of serum citrate concentrations are much too low, however, to indicate absorption of calcium as a calcium citrate complex. The possibility remains, however, that citrate at the surface of the cells may play a role in the uptake and transport of calcium by the mucosal cell. Strontium feeding unlike calcium does not produce a rise of serum citrate levels (fig. 7).

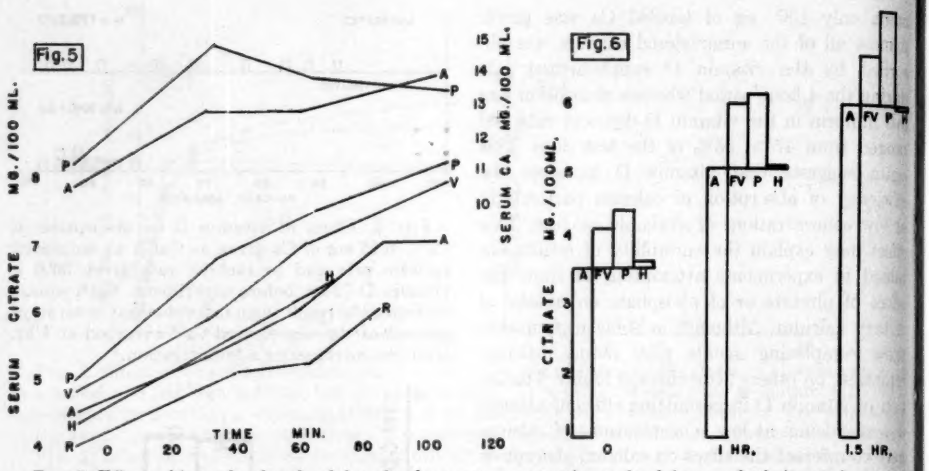


FIG. 5. Effect of intraduodenal calcium load upon concentrations of calcium and of citrate in portal blood of dog. The upper pair of lines show the concentrations of calcium in arterial and portal serum. The group of lines in the lower part of the graph show the concentrations of citrate in serum following introduction of calcium lactate solution intraduodenally at 0 time. A—arterial, P—portal, V—femoral venous, H—hepatic venous, R—renal venous.

FIG. 6. Average concentrations of citrate in arterial serum and arterio-venous differences following intraduodenal calcium lactate administration to dogs. The data given are the average of 6 experiments and are plotted as in fig. 4.

FIG. 7. Lack of effect of strontium administration upon citrate concentrations in arterial and portal venous serum of dog.

Cortisol reduces serum citrate levels and prevents in rats the citrate raising effect of calcium loading (fig. 8). In more recent studies the effect of cortisol appears to be due to its inhibitory action on the efflux of citrate from cells to extracellular fluid and not on the intracellular accumulation of citrate (10). This cortisol action is mentioned because adrenocortical steroids have been found to decrease calcium absorption from the intestine. This effect is perhaps more striking in patients with abnormally high rates of intestinal absorption of calcium e.g. patients with sarcoidosis (11), hypervitaminosis D and idiopathic hypercalcemia (12), than in normal individuals. Whether the effect of vitamin D and of cortisol upon citrate levels in body fluids have any causal connection with their action on calcium absorption or is coincidental, remains to be demonstrated.

There are a number of other factors which influence the efficiency of calcium absorption from the gut. The effect of high protein diet in increasing calcium absorption has been noted, and more detailed experimental studies by Wasserman and his colleagues (13, 14) have indicated that the amino acids lysine and arginine are particularly active in promoting calcium absorption. No explanation for this phenomenon is forthcoming although it has been suggested that these amino acids may influence the transport mechanism for calcium in the mucosa. Calcium absorption has also been noted to be increased by high lactose intake. On the other hand disturbances of intestinal motility, or impairment of overall intestinal absorption of fat will interfere with absorption of calcium. The long term studies of Malm (15) on prisoners have indicated that emotional disturbances may interfere with intestinal absorption of calcium in the absence of obvious diarrhea.

I have already referred to the regulation of calcium absorption by the intestine as a homeostatic mechanism. What is the evidence that intestinal absorption varies according to a pattern which allows for adequate absorption of calcium when required, but prevents excessive absorption and excessive loads for excretion in the urine

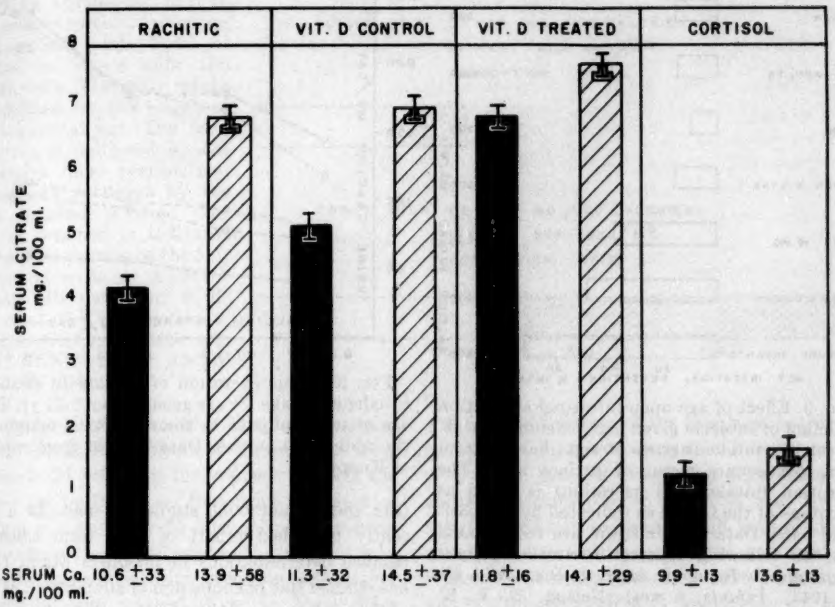


FIG. 8. Effect of cortisol treatment in rats upon serum citrate concentrations with and without calcium loading. In the last pair of columns the serum citrate levels of cortisol fed rats are seen to be much lower than those of rachitic or of vitamin D treated rats and no increase of citrate results from intraintestinal calcium administration.

when calcium is not required or can not be deposited as bone salt?

There is first the age effect or the correlation of efficiency of calcium absorption with growth. Young growing animals absorb dietary calcium much more completely than older animals. This effect has been demonstrated in a variety of animals (16, 17). Figure 9 indicates that this age effect is also found in man. The net intestinal retention i.e., the difference between intake and fecal loss is shown as percentage of the intake for the span of life from the prematurely born infant to the aged man. The data have been collected from many sources but in all of these metabolism studies the subjects have been ingesting a high calcium intake, with a calcium to phosphorus ratio in the range of usual diets, and in addition have been given adequate amounts of vitamin D. The comparison of calcium intakes for subjects of such different sizes has little theoretic basis, but for this purpose calcium intake has been expressed per square meter of surface area. The high absorptive efficiency in the premature infant group, and the infants during the first year of life is evident. There is a rapid drop in later childhood and in adult life. There is little difference in degree of absorption between the older child and the adult and in this respect the studies of Bronner and Harris (18) using radioactive Ca are in agreement with the results of balance studies. These heterogenous studies presented in this figure would not be of significance per se but they are in general agreement with the findings in experimental animals which show that the completeness of absorption of dietary calcium diminishes rapidly with increasing age. However, age or growth rate alone is not necessarily the controlling factor. There is evidence that the state of the skeleton or the degree to which calcium can be deposited in skeletal matrix in some way regulates calcium absorption. For example, studies of osteomalacic adults by Liu and co-workers (19) have shown that following vitamin D therapy and induction of rapid bone salt deposition in osteomalacic bone, the net intestinal retention of calcium approached the values found in young infants, namely over 70% of the intake. Similarly Nicolaysen (20) has found in rats that the intestinal uptake of calcium was greater in old rats who had previously been depleted of calcium than in animals of the same age with skeletons of normal bone salt content. Nicolaysen has

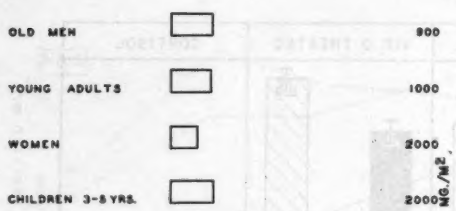


FIG. 9. Effect of age upon intestinal absorption of calcium of subjects given high calcium intakes. The calcium intake is given at right-hand margin as mg per square meter of surface area. The absorption (intake–fecal excretion) is given as percentage of the intake as indicated by the horizontal bars. Data in this figure are recalculated from the following reports: *Premature Infants* (Benjamin, H. R., *et al. Amer. J. Dis. Child.* 65: 412, 1943). *Infants, 6 mos.* (Nelson, M. V. K. *Amer. J. Dis. Child.* 42: 1090, 1931). *Infants, 10 mos.* (Nelson, M. V. K. *Amer. J. Dis. Child.* 42: 1090, 1931). *Children,* 3–5 yr. (Daniels, A. L., *et al. Amer. J. Dis. Child.* 47: 400, 1934). *Pregnant women* (Hummel, F. C., *et al. J. Nutrition* 11: 235, 1936). *Young adults* (Leichenspring, J. M., *et al. J. Nutrition* 45: 407, 1951). *Old men* (Bogdonoff, M. D., *et al. J. Gerontol.* 8: 272, 1953).

FIG. 10. Urine excretion of calcium in relation to calcium intake for age groups from 1–24 yr. The age groups are given at the right hand margin of the individual curves. Data plotted from report of Knapp (24).

coined the term 'endogenous factor' suggesting by implication that some substance is elaborated in states of skeletal demineralization which influences absorption of calcium from the intestine. The nature of such a factor or the site of elaboration are not suggested. At the moment this mechanism must be considered as unknown, but it is apparent that the uptake of calcium by the intestine is in some way influenced by the rate of deposition of bone salt in the skeleton, or the state of mineralization of the bone.

The effect of the level of calcium intake on efficiency of absorption of calcium has also been given much attention. Subjects maintained continuously on low calcium intakes absorb a higher proportion of the dietary calcium than subjects who have been given higher levels of calcium and then are changed to the low calcium diets. It has been proposed that an adaptation to low calcium diets occurs which results in increased efficiency of calcium absorption. Such adaptation has been observed in studies on rats by Henry and Kon (21) and in metabolic studies in man. In a recently published report of long term calcium balance determinations in prisoners Malm (15) has studied this phenomenon of adaptation. Most of the subjects studied by him showed adaptation in the sense of progressive decrease of fecal excretion of calcium when changed from moderate to low calcium intakes. In a small group of subjects adaptation did not occur and the fecal calcium remained high relative to the dietary intake. The time required for development of such adaptation in subjects who did adjust was variable. Hegsted *et al.* (22), Walker (23), and others have stressed the ability of man to adapt to diets low in calcium by increased efficiency of intestinal absorption of calcium which in their mind accounts for the absence of demonstrable calcium deficiency states in parts of the world where the diet is uniformly low in calcium according to our standards.

When large intakes of calcium are given the percentage of the intake which is rejected by the intestine is increased so that the amount absorbed is not proportional to intake. Whether lack of absorption of the excess calcium can be considered as an adaptive reaction is not known. The factors which act to limit calcium absorption in so far as intestinal function is concerned have not been given much attention with the exception of the effect of agents which reduce calcium absorption by complexing with calcium. As a result of the limited absorption of calcium at high intakes the urine calcium output is not increased in proportion to intake. Figure 10 shows data from Knapp's

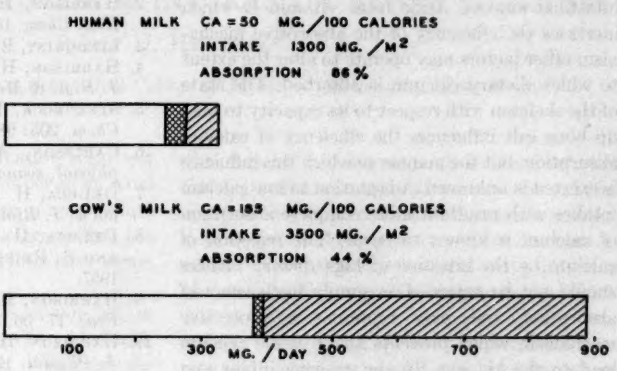


FIG. 11. Comparison of fecal and urinary calcium excretions and calcium retentions of infants fed cow's milk and human milk. The daily intake is indicated by the length of the horizontal bar. The fecal excretion is indicated by the diagonally lined portion and the urinary excretion by the cross hatched portion. The calcium retained is indicated by the clear portion of the bar. All data given as mg per 24 hr. Human milk data from S. H. Liu, *et al. J. Clin. Invest.* 19: 333, 1940. Cow's milk data from M. V. K. Nelson, *Am. J. Dis. Child.* 42: 1090, 1931.

(24) study of urine excretion of calcium in relation to intake for subjects of varying ages from 1 year to 24 years. In the younger subjects the curves are fairly flat so that urine excretion of calcium is raised only 25–30% despite a several fold increase in calcium intake. In the older subjects the increase in urine excretion with higher intakes is more marked. Even in these older individuals the rejection of calcium at high levels of intake is sufficient to prevent intolerable loads of calcium as far as the kidney is concerned. There is, however, marked individual variation in this response.

The adaptation of subjects to high calcium diets is illustrated by the extensive experience with feeding of cow's milk to infants. The calcium content of human milk is 0.5 mg per calorie whereas that of cow's milk is 1.8 mg per calorie. As shown in figure 11 infants fed cow's milk absorb a smaller percentage of the intake than do infants given human milk although the absolute amount of calcium absorbed is greater in the former. Urine output of calcium is about the same in the two groups of infants. The actively growing infant can presumably adapt to high calcium intakes by increased uptake of calcium by the skeleton in addition to limitation of absorption of dietary calcium. In the adult subject the uptake of calcium by mature non growing bone is not quantitatively as important, so that rejection of calcium by the intestine is the only protective mechanism. The factors governing this aspect of regulation of intestinal absorption of calcium are not known and will probably not be understood until more is learned of the fundamental processes which determine the transport of calcium across the intestinal mucosa.

Failure of function of the intestinal barrier to calcium, or in more conventional terms excessively high rates of absorption of calcium, can occur in certain disease states so that hypercalcemia and hypercalciuria result with resultant metastatic calcification and renal and vascular injury. These states thus resemble hypervitaminosis D although vitamin D dosage may not have been excessive. One such entity is 'idiopathic' hypercalcemia of infants which has been recognized only since World War II. Most of the reports have come from England and Scotland and the problem appears to result from the combination of high calcium intake of cow's milk feeding in infancy with moderately high intakes of vitamin D in the form of fortified milk and cereals as well as vitamin D concentrates. This type of feeding is obviously well tolerated by the vast majority of infants so fed, and the hypercalcemia seen in the rare patient must represent some individual abnormality of metabolism. One possibility which has been suggested is a failure of the inactivation or elimination of vitamin D so that excessive tissue concentrations of vitamin D result despite ordinarily non-toxic doses (25). Whether this is also the mechanism in patients with sarcoidosis or pulmonary carcinoma with hypercalcemia is still unsettled. At any rate these particular subjects can not tolerate high calcium intakes since an excessive amount of calcium is absorbed. The protective mechanism of incomplete intestinal absorption of calcium can be inadequate under these conditions.

In summary, the absorption of calcium depends both upon the availability i.e. the concentration of calcium ion in intestinal contents and the efficiency of the transport mechanism in the

intestinal mucosa. Aside from vitamin D which increases the efficiency of the absorptive mechanism other factors may operate to alter the extent to which dietary calcium is absorbed. The state of the skeleton with respect to its capacity to take up bone salt influences the efficiency of calcium absorption, but the manner in which this influence is exerted is unknown. Adaptation to low calcium intakes with resultant more complete absorption of calcium is known to occur. The rejection of calcium by the intestine at high dietary intakes should not be regarded as simply inefficiency of absorption, but may represent a protective mechanism which prevents an excessive calcium load to the kidneys. In the growing infant and child, adjustment to high calcium intakes is possible with little increase in urinary calcium. In the adult this adjustment is perhaps less complete. Even in the infant excessive absorption of calcium may result if vitamin D intake is above the necessary maintenance level. It is possible that in the adult, also, excessive absorption of calcium in certain disease states may be related to increased vitamin D activity in the body. If diets high in calcium are taken it is probably desirable to limit vitamin D intake to the quantities needed for maintenance which are considered to be 400 units per day in the child and probably of the same order of magnitude in the adult. At the present time it must be concluded that man can adjust to a wide range of calcium intakes. Under certain circumstances excessive amounts of calcium are absorbed but this probably involves factors other than simply high calcium intake.

REFERENCES

1. Nicolaysen, R., N. Eeg-Larsen and O. J. Malm, *Physiol. Rev.* 33: 424, 1953.
2. Harrison, H. E. and H. C. Harrison, *J. Biol. Chem.* 185: 857, 1950.
3. Lindquist, B. *Acta Paediat.* Suppl. 1, 1952.
4. Harrison, H. E. and H. C. Harrison. *Yale J. Biol. & Med.* 24: 273, 1952.
5. Steenbock, H. and S. A. Bellin. *J. Biol. Chem.* 205: 985, 1953.
6. Carlsson, A. and G. Hollunger. *Acta physiol. scandinav.* 31: 317, 1954.
7. DeLuca, H. F., F. C. Gran and H. Steenbock. *J. Biol. Chem.* 224: 201, 1957.
8. DeLuca, H. F., F. C. Fan, H. Steenbock and S. Reiser. S. *J. Biol. Chem.* 228: 469, 1957.
9. Harrison, H. C. and H. E. Harrison. *Fed. Proc.* 17: 66, 1958.
10. Harrison, H. C. and H. E. Harrison. *Am. J. Physiol.* 196: 943, 1959.
11. Anderson, J., C. E. Dent, C. Harper and G. R. Philpot. *Lancet* 2: 720, 1954.
12. Morgan, H. G., R. G. Mitchell, J. M. Stowers and J. Thomson. *Lancet* 1: 925, 1956.
13. Wasserman, R. H., C. L. Comar and M. M. Nold. *J. Nutrition* 59: 4025, 1956.
14. Wasserman, R. H., C. L. Comar, J. C. Schooley and F. W. Langamann. *J. Nutrition* 62: 367, 1957.
15. Malm, O. J. *Scandinav. J. Clin. & Lab. Invest.* 10: Suppl. 36, 1958.
16. Hansard, S. L. and H. M. Crowder. *J. Nutrition* 62: 325, 1957.
17. Hansard, S. L., C. L. Comar and M. P. Plumlee. *J. Animal Sci.* 13: 25, 1954.
18. Bronner, F. and Harris, R. S. *Ann. New York Acad.* Sc. 64: 314, 1956.
19. Liu, S. H., H. I. Chu, H. C. Hsu, H. C. Chao and S. H. Cheu. *J. Clin. Invest.* 20: 255, 1941.
20. Nicolaysen, R. *Acta physiol. scandinav.* 5: 200, 1943.
21. Henry, K. M. and S. K. Kon. *Brit. J. Nutrition* 7: 147, 1953.
22. Hegsted, D. M., I. Moscosco and C. C. Collazos. *J. Nutrition* 46: 181, 1952.
23. Walker. A. R. P. *Metabolism* 3: 114, 1955.
24. Knapp, E. L. *J. Clin. Invest.* 26: 182, 1947.
25. Fellers, F. X. and R. Schwartz. *New England J. Med.* 259: 1050, 1958.

EFFECTS OF HIGH CALCIUM INTAKES ON RENAL FUNCTION

PHILIP H. HENNEMAN

Department of Medicine, Seton Hall College of Medicine, Jersey City, New Jersey

THERE IS LITTLE direct information regarding the effects of high calcium intakes per se on renal function. However, the occurrence of renal failure in various hypercalcemic states suggests that the dietary requirements for calcium may have upper as well as lower optimum limits.

Most data on calcium intakes of one gm or more per day are based on diets with added milk which contains roughly 1000 mg of calcium per liter. However, milk also contains abundant phosphorus, protein and other factors which affect the absorption and metabolism of calcium. Now that vitamin D is added to virtually all milk in the United States one cannot even exclude this potent calcium regulator from discussions of high calcium intakes.

Perhaps the least complicated way to study the effects of high calcium intakes is with intravenous calcium administration. Under such circumstances one can measure the amount given, the resultant calcium concentrations in the serum and the duration of the effect. However, most studies of calcium infusions are of short duration. By contrast, calcium administered orally enters the body in proportion to the dose administered but only as facilitated by parathyroid hormone, vitamin D, growth (1), thyroid and sex hormones. Calcium absorption probably is increased by concomitant high protein and high phosphorus intakes (2) and decreased by systemic acidosis. The exact biochemical mechanism of calcium absorption is the first unknown to which attention is drawn. Studies of oral calcium intakes are nearly always chronic in duration.

Calcium which enters the circulation is distributed rapidly throughout the serum and extracellular fluid and is disposed of by intestinal secretion (which appears independent of serum calcium levels), by entrance into bone and into the hydration shell about bone, and by urinary excretion which varies directly with the level of serum calcium. In addition, some calcium enters the soft tissues, probably in proportion to the blood level of calcium. Therefore hypercalcemia is an index of the various rates of calcium entrance into the circulation and exit from it.

Numerous investigators (3–7) have studied the acute effects in man of intravenous calcium infusions which produce mild hypercalcemia for 4–24 hours. They report a prompt increase in urinary phosphate excretion and a rise in serum phosphorus. This probably represents not inhibition of parathyroid function as originally suggested, but a direct effect of hypercalcemia on soft tissue release of phosphorus, since the serum phosphorus rises following calcium infusions even in hypoparathyroid subjects. Calcium infusions also produce a slightly delayed rise in urinary calcium, an increase in urinary sodium and potassium excretion, and an increase in water excretion. These latter changes appear to be direct though poorly understood effects of hypercalcemia on renal function. Yoffee and Dingman (8) have recently reported that brief episodes of hypercalcemia and hypercalciuria do not alter the physiologic effects of vasopressin on water conservation. No harmful effects of such brief episodes of hypercalcemia have been reported.

Sanderson (9) has recently reported studies in rats on acid, high calcium, high phosphorus diets[1] which produced significant loss of maximum urinary concentrating capacity within one week and reduction of maximum urinary osmolality to 60% of control values within three weeks. This study is of special pertinence in that these animals showed no rise in serum calcium or phosphorus and no change in blood urea concentration. Sanderson further showed that the kidneys of such calcium-intoxicated rats might or might not demonstrate histological evidence of slight calcification in the region of the collecting tubules but did show a 300-fold increase in renal calcium content. Although there was some recovery of renal concentrating ability on restoration of a lower calcium intake the renal calcium content did not fall—showing this to be basically an irreversible lesion. Similar studies are needed in man; however, the apparently irreversible nature of the renal calcification observed by Sanderson

[1] *Diet*: sucrose, 100 parts; dried milk 100 parts; calcium phosphate ($Ca_3(PO_4)_2$), 10 parts; 85% phosphoric acid, 10 parts.

emphasizes the potential danger of such an investigation in man.

Epstein (10) has carried out similar experiments in rats using massive doses of vitamin D[2] to produce calcium intoxication. He observed loss of concentrating ability by the kidneys in all animals while only a few animals demonstrated urea retention or even slight decreases in PSP excretion. Morphological lesions in the kidneys were sparse, and involved primarily the collecting tubules. Loss of renal concentrating ability in such experiments may be the consequence of loss of this one specific function (as occurs in nephrogenic diabetes insipidus (11)) or, less likely, depression of all functions of many whole nephrons which would then impose a solute diuresis on the remaining intact tubules.

In man, toxic effects commonly attend the hypercalcemia of rapid bone destruction by metastatic carcinoma or by severe hyperparathyroidism. At present hypercalcemia appears to be the major toxic factor in these states and it is assumed that most of the clinico-pathologic picture is due to hypercalcemia. Slight hypercalcemia usually produces no clinical signs, does not affect renal function as measured to date and is detected only on chemical investigation of the patient with osteitis fibrosa generalisata or renal stones. Moderate hypercalcemia may be asymptomatic but often produces slight constipation, polyuria and polydipsia in addition to effects on bones and on stone-formation. The earliest sign of renal dysfunction in hyperparathyroidism is usually polyuria due to decreased concentrating capacity. At this stage renal architecture may be normal or may show slight to moderate nephrocalcinosis with deposits of calcium phosphate within collecting tubules, in the basilar portion of cells in this area or in the interstitial tissue about collecting tubules. This decrease in urine-concentrating capacity may appear after only several weeks of hypercalcemia and often is entirely reversible. Some patients also show decreased ability to excrete PSP dye.

More severe hypercalcemia may be associated with depression of all renal functions and may elicit mild albuminuria with casts of amorphous calcium phosphate but without pyuria or hematuria. If hypercalcemia is promptly reversed, marked recovery of renal function may occur; if hypercalcemia has been prolonged, renal function may cease deteriorating but usually does not improve significantly. In addition, chronic hypercalcemia, especially if associated with alkalosis, leads to suppression of sweating, hyperkeratinization of skin with generalized pruritus, calcification of cornea and conjunctiva with symptoms of ocular irritation, and metastatic calcification, particularly in the fat about joints.

When the serum calcium reaches 17–20 mg % death frequently occurs within several weeks due to renal failure. At autopsy, such patients or experimental animals show metastatic calcification, particularly of the falx of the brain, gastric mucosa, thyroid, lung, myocardium, and kidney. All these changes may be reproduced in laboratory animals by injections of large doses of parathyroid extract. Albright coined the phrase 'hyper-hyper-parathyroidism' to describe the sudden rise in serum phosphorus, failure of renal function with rise in NPN and death which occurred with extremely large doses of parathyroid extract in puppies.

Excessive intake of vitamin D regularly produces hypercalcemia followed by depression of renal function and uremia. The dose of vitamin D responsible for overt poisoning is usually in excess of 200,000 units daily although doses as small as 6000 units daily may poison patients with concomitant sarcoid (12).

The milk-alkali syndrome first described by Burnett *et al.* (13) represents the occurrence of hypercalcemia, alkalosis and uremia in patients on large intakes of milk and absorbable alkali. It remains obscure why more ulcer patients treated with similar calcium intakes do not develop this picture; what is the contribution of the absorbable alkali, and why is it possible that the hypercalcemia may persist long after the milk intake ceases. In regard to the latter problem Dr. William H. Baker and I (14) did not observe increased calcium absorption but did observe greatly increased radio-calcium exchangeable pool size in two patients recovering from the milk-alkali syndrome. Since their metastatic calcification contained calcium with high specific activity we concluded that soft tissue calcium was exchanging with blood calcium at a rapid rate and was responsible for maintaining the mild hypercalcemia.

The problem of varying capacity to absorb calcium and varying sensitivity to vitamin D re-

[2] Diet used was Purina Lab Chow with 200,000 to 400,000 IU vitamin D in peanut oil injected subcutaneously each day for 4 days.

mains a major riddle. Although hyperadrenocorticism may reduce the hypercalcemic response to vitamin D, this does not always occur.

In recent years Lightwood (15), Fanconi (16) and others have called attention to the occurrence of hypercalcemia, renal failure, dense bones and failure to thrive in certain infants fed cows' milk and 1800–4000 units of vitamin D daily. This abnormality appears soon after birth and if the children survive, they gradually recover spontaneously. Current information (17, 18) indicates that the syndrome is the consequence of hypercalcemia resulting from excessive calcium absorption probably due to what previously was not recognized as excessive intakes of calcium and vitamin D. The condition is ameliorated by low calcium intakes, cortisone and sodium phytate. Generalized depression of all renal functions is the rule in these patients. To what extent phosphorus metabolism, direct effects of vitamin D on the kidney, and fatty acid metabolism, may contribute to the clinico-pathologic picture is not known.

Approximately 30% of patients with sarcoid develop hypercalcemia with increased calcium absorption suggestive of hypervitaminosis D (19). Decreased glomerular filtration, decreased capacity to conserve water and decreased capacity to excrete PSP dye are common in such patients. Howard *et al.* (20) were not able to demonstrate increased vitamin D-like activity in the serum of such patients although they pointed out that the biological assay used would not detect some closely related congeners of vitamin D_2 such as dihydrotachysterol. The hypercalcemia in sarcoid does not appear to be related to bone involvement and may be suppressed with corticoid therapy or sodium phytate. In my experience the liver has always been involved by sarcoid in those patients who present hypercalcemia; however not all patients with liver involvement present hypercalcemia.

The effort to relate hypercalcemia in sarcoid and in infant hypercalcemia to vitamin D calls attention to the paucity of information regarding vitamin D metabolism in man. Especially needed are improved chemical methods for measurement of vitamin D_2 and D_3, isotope-labeled vitamin D and further use of labeled vitamin D in experiments concerning the absorption, fate and excretion of vitamin D.

It has been suggested (21) that high milk intakes may produce renal stones as a consequence of hypercalciuria. Although high intakes may be associated with hypercalciuria the relationship is not very consistent or direct (22). Liu, Su, Chou, Chu, Wang and Chang (23) have presented data relating calcium intake and urinary calcium of a patient studied at several levels of calcium intake and several levels of phosphorus intake as well. Urinary calcium rose with each increment of calcium intake, but decreased with each increment in phosphorus intake. Since high calcium intakes usually represent high phosphorus intakes as well one can appreciate the necessarily complex relationship of calcium intake to urinary calcium.

My final data concerns the occurrence of renal stones in patients drinking upwards of one quart of milk daily. In my experience this is a not uncommon relationship. Thus, of 207 consecutive patients studied in a special clinic for kidney stone patients at the Massachusetts General Hospital, the most likely cause of stones in nine (4%) was excessive milk intake (21). The urinary calcium of these patients is decreased by omitting milk from their diet and in general they do not form further stones after discontinuing use of milk. The relationship has been observed often enough so that I believe a high milk intake is a common cause for renal stones. However, no one has yet presented data from a large series of subjects regarding the effect on urinary calcium of adding one quart of milk daily to the diet. Perhaps only certain individuals develop hypercalciuria and renal stones on high calcium intakes.

It is difficult to summarize these loosely related points. On the one hand it appears that hypercalcemia commonly depresses renal function whatever the cause of the hypercalcemia and regardless of the level of serum phosphorus or other variables. Both human and rat studies suggest that urinary concentrating capacity is the renal function first depressed by hypercalcemia. In at least one study in rats high calcium intakes depressed this function without producing hypercalcemia or hyperphosphatemia. The increase in renal calcium appeared to be irreversible.

On the other hand, the value of most of the experimental data is reduced because possible effects of concomitant parathyroid hormone, vitamin D, or other dietary manipulations have not been excluded.

It appears likely that high calcium intakes may

affect renal function unfavorably but more information is needed to specifically indict calcium as well as to clarify the metabolism of vitamin D and the intestinal and renal handling of calcium. Finally cellular and isolated enzyme studies are needed to clarify the mechanism by which calcium depresses renal function.

REFERENCES

1. Henneman, P. H., A. P. Forbes, M. Moldawer, E. F. Dempsey and E. L. Carroll. Submitted for publication.
2. Henneman, P. H. Unpublished data.
3. Baylor, C. H., H. E. Van Alstine, E. H. Keutman and S. H. Bassett. *J. Clin. Invest.* 29: 1167, 1950.
4. Howard, J. E., T. R. Hopkins and T. B. Connor. *J. Clin. Endocrinol.* 13: 1, 1953.
5. Chambers, E. L., G. S. Gordan, L. Goldman, and E. C. Reifenstein, Jr. *J. Clin. Endocrinol.* 16: 1507, 1957.
6. Nordin, B. E. C. and R. Fraser. *Clin. Sc.* 13: 477, 1954.
7. Lichwitz, A., S. DeSeze, D. Hioco and P. Borcher. *Sem. Hôp. Paris* 31: 3789, 1955.
8. Yoffee, H. F. and J. F. Dingman. *Clin. Res.* 7: 272, 1959.
9. Sanderson, P. H. *Clin. Sc.* 18: 67, 1959.
10. Epstein, F. H., M. V. Rivera and F. A. Carone. *J. Clin. Invest.* 37: 1709, 1958.
11. Waring, A. J., L. Kajdi and V. Tappan. *Am. J. Dis. Child.* 69: 327, 1945.
12. Larsson, L. G., A. Lilijestrand and H. Wahlund. *Acta med. scandinav.* 143: 180, 1952.
13. Burnett, C. H., E. R. Commons, F. Albright and J. E. Howard. *New England J. Med.* 240: 787, 1949.
14. Henneman, P. H. and W. H. Baker. *J. Clin. Invest.* 36: 899, 1957.
15. Lightwood, R. *Proc. Roy. Soc. Med.* 45: 401, 1952.
16. Fanconi, G., P. Girardet, B. Schlesinger, N. Butler and J. Black. *Helvet. paediat. acta* 7: 314, 1952.
17. Stapleton, T., W. B. MacDonald and R. Lightwood. *Lancet* 1: 932, 1956.
18. Fellers, F. X. and R. Schwartz, *New England J. Med.* 259: 1050, 1958.
19. Henneman, P. H., E. F. Dempsey, E. L. Carroll and F. Albright. *J. Clin. Invest.* 25: 1229, 1956.
20. Thomas, W. C. and J. E. Howard. *J. Clin. Invest.* 38: 1078, 1959.
21. Melick, R. A. and P. H. Henneman. *New England J. Med.* 259: 307, 1958.
22. Knapp, E. L. *J. Clin. Invest.* 26: 182, 1947.
23. Liu, S. H., C. C. Su, S. K. Chou, H. I. Chu, C. W. Wang and K. P. Chang. *J. Clin. Invest.* 16: 603, 1937.

EFFECTS OF HIGH CALCIUM INTAKES ON URINE IN ANIMALS[1]

C. W. Vermeulen

Department of Surgery, University of Chicago, Chicago, Illinois

For the past 10 years our laboratory has been engaged in a study of urinary stone disease using as a test object the rat with a foreign body in the bladder (1). In clinical experience it is not uncommon to find that a foreign body, when present in the urinary system, stimulates the formation of stone, the foreign body acting as the nucleus about which the stone forms. We have merely employed this well-known phenomenon to make as it were, a laboratory model of stone disease. Admittedly the model is an imperfect and artificial one, but for experimental purposes it has been found quite convenient.

[1] These investigations were supported by grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health.

METHODS

Customarily a small disc of zinc is used as the standard foreign body, though a variety of other materials would serve equally well in stimulating stone formation. The zinc discs, 4 mm in diameter, are sterilized and placed in the urinary bladder by open surgical operation. The rats are given a diet of Purina Laboratory Chow which is not calculogenic in intact rats. However, if a foreign body be present, stones consistently form within a period of 6 weeks. The rats are then operated upon once again, the bladder exposed, and urine aspirated aseptically for pH determination and bacterial culture. Previous observations (2) have shown that infection with many different organisms may significantly alter the amount of stone produced in experiments of this type.

We therefore believe it is important to discard the results from any infected animal in order to avoid erroneous conclusions due to accidental infection. After urine has been aspirated for culture and pH, the bladder is opened and the stone removed, weighed, and the weight of the foreign body subtracted. Thus the result, stone growth, is expressed quantitatively as stone weight. Average stone growth for any group of rats is computed after first eliminating those rats that yielded a positive urine culture. Group comparisons are then made and statistically evaluated.

Soon after this project began, it was observed that different rats may make stones of different chemical composition, and that both sex and genetic strain of the rat play a role in determining the chemical nature of the stone (3). This curious phenomenon need not be considered further except to say that 2 types of rats are regularly used in our experiments. One type consistently makes stones composed of pure magnesium ammonium phosphate hexahydrate; the other type of rat produces stones composed of calcium phosphate even though the rats are given the identical diet of Laboratory Chow.

EFFECT OF CALCIUM ADDITION TO THE DIET

With this general background, an experiment may now be considered where we tried to make calcium stones in the type of rat that ordinarily makes magnesium stones. We thought that if the normal diet were fortified with extra calcium (4), the urinary calcium would rise and calcium stones might now form instead of the usual magnesium variety. So calcium lactate was added to the Laboratory Chow in amounts sufficient to double its calcium content, lactate being selected as a palatable and easily absorbed form of calcium. Control animals on the normal diet were studied simultaneously, all animals having zinc foreign bodies in the bladder.

Six weeks later, unexpected results were obtained, as shown in figure 1. The control group, receiving the normal diet, consisted of the 25 rats having a sterile urine at the end of the experiment. The average stone weight for the group was 60 mg, a customary amount as judged from previous experiments of this sort. The stones were composed of magnesium ammonium phosphate. A like number of rats were in the group that received the diet containing the extra calcium. In this group, where we had expected that stones of the calcium type might form, very little stone growth occurred.

The experiment was now repeated using the other kind of rat, the kind which ordinarily produces calcium stones. The results of this experiment are shown in figure 2. The control group of 27 rats developed the usual amount of stone (calcium variety) while the group receiving the diet fortified with calcium again showed stone impedience. We are confronted, then, with an apparent paradox, for feeding extra amounts of one of the stone components inhibited stone formation. This does not seem to make sense.

In search of an explanation, urine collections were made for 24-hour periods at intervals throughout the experiment using special metabolism cages with 2 rats per cage. The values mentioned here were derived from a minimum of 20 separate collections in each rat group. Figure 3 shows some of the urine findings in the experiment just mentioned with the rats that ordinarily develop stones of the calcium variety. Since the urine volume did not vary significantly, the data, though expressed as urine concentrations, also indicate the approximate relative per diem excretions. The urinary calcium rose to a very high level when calcium was added to the diet while simultaneously a reciprocal fall in phosphorus occurred. On the other hand, the change in citrate paralleled that of calcium, for the citrate concentration rose to four times the normal value. Magnesium excretion, not shown on the slide, remained relatively unchanged.

However, the rats fed calcium lactate developed a slightly alkaline urine. The average pH of the control group was 6.7 while an average pH of 7.3 was obtained for the experimental group. Of course the alkalinity of the urine will not explain the observed stone inhibition since the stone salt is actually more insoluble in alkaline media, but perhaps the acid-base alteration accounts for some of the urine changes, such as

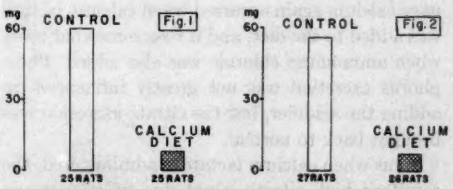


FIG. 1. Average stone weight. Stone growth on foreign bodies in period of 6 weeks. Rats used here make magnesium ammonium phosphate stones on control diet.

FIG. 2. Average stone weight. Stone growth using rats that make calcium phosphate stones on control diet.

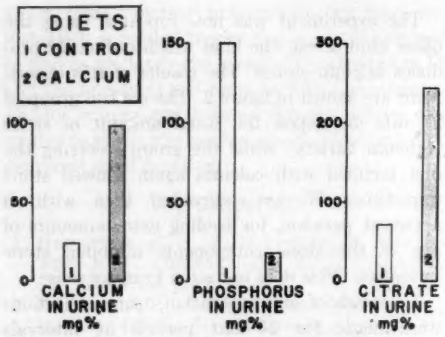


FIG. 3. Urine concentrations. Urine chemistry in animals of experiment shown in fig. 2.

the rise in citrate. It is known that citrate excretion, in general increases with increasing alkalinity.

Another experiment was therefore done in which we attempted to counteract the alkalinizing effect of calcium lactate. Since we had had previous experience with ammonium chloride as an acidifier, it was used in an effort to neutralize the pH effect of calcium lactate. We did not know the amount of ammonium chloride that would be necessary to produce the desired effect, so three different dose levels were used: 0.5, 1.0 and 1.5% ammonium chloride added to the calcium lactate diet. The 1.0% dose was found to be the proper amount since with it the urine pH was almost exactly the same as the pH of the controls receiving only the normal Laboratory Chow.

Figures 4 and 5 show the result of this new experiment. Once again the expected amount formed in a control group receiving the normal diet, and once again very little stone occurred in the group receiving calcium lactate. In the third group, receiving both extra calcium and ammonium chloride, stone formation was still impeded. As indicated in figure 5, the rise in urinary calcium again occurred when calcium lactate was added to the diet, and it rose somewhat more when ammonium chloride was also added. Phosphorus excretion was not greatly influenced by adding the acidifier, but the citrate excretion was brought back to normal.

Thus when calcium lactate is administered, the resultant high citrate is not due to calcium per se, but to the coincident alkalinization. When the pH change was prevented by simultaneously giving ammonium chloride, the citrate excretion returned to normal though stones still failed to form. Obviously, citrate changes do not explain the stone prevention observed when extra calcium was fed. Rather, we believe that the stone impedience observed in these experiments was due to the marked reduction in urinary phosphorus. Apparently, the phosphorus reached a critical level below which calcium phosphate stones cannot form even though the calcium concentration is unusually high.

EFFECT OF ADDING PHOSPHORUS TO THE DIET

Reverse experiments have also been done in which extra phosphate was added to the diet instead of extra calcium (5). These experiments may be mentioned briefly to further illustrate the reciprocal relations between calcium and phosphorus. By doubling the dietary phosphate the urinary phosphate rose to high levels with a simultaneous fall in urine calcium. Again stones were prevented. Three different phosphates have been studied in this way; first, ordinary orthophosphate salt; second, sodium hexametaphosphate (the commercial water softener Calgon);

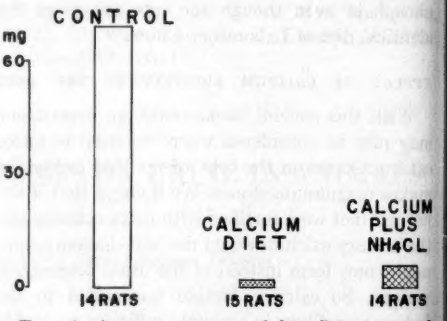


FIG. 4. Average stone weight. Stone growth using rats that make calcium phosphate stones on control diet.

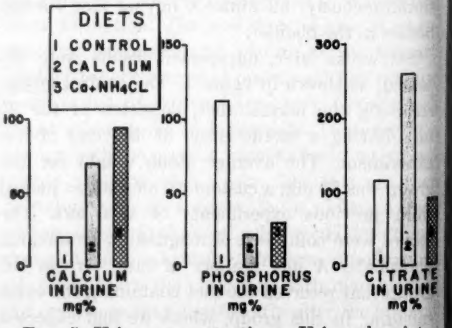


FIG. 5. Urine concentrations. Urine chemistry in animals of experiment shown in fig. 4.

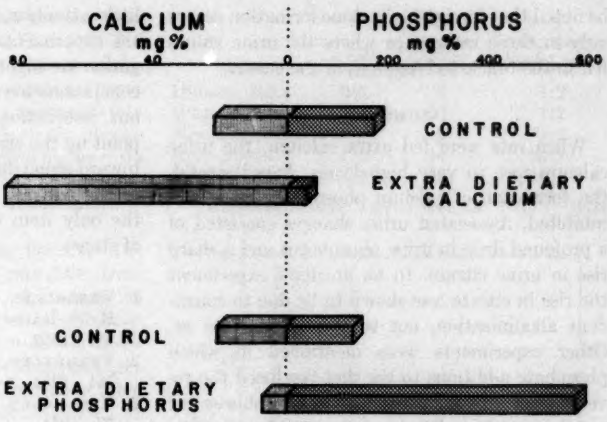


FIG. 6. Urine concentrations. Shift of urinary calcium and phosphorus when extra amounts of one of these ions are added to the diet.

and third, sodium phytate (which by its structural formula is essentially a benzene ring with 6 phosphate radicals attached). All 3 of these substances performed equally well when given in comparable dosage calculated for phosphate content. All 3 produced the same urine changes and the same stone impedience. Thus in these experiments no special hexametaphosphate or phytate action was found since phosphate as the ordinary ortho salt served equally well.

RECIPROCAL SHIFT OF URINE CALCIUM AND PHOSPHORUS

It appears, then, that dietary addition of phosphate results in a marked decrease in urine calcium, while dietary addition of calcium produces a reverse effect with a fall in urine phosphorus. Figure 6 attempts to illustrate these reciprocal relations by comparing the effect of normal diets and diets containing twice the normal amounts of either calcium or phosphorus.

In the course of our study a number of experiments have by now accumulated in which varying amounts of calcium or phosphate were added to the diet in an attempt to shift the urine calcium and phosphate to greater or lesser extent. In figure 7 the average urine values have been plotted against each other, using all the data accumulated so far on the type of rat which ordinarily forms calcium phosphate stones. Each observation on the graph represents the average urine values from a group of 15 or more rats, plotting the urine calcium in mmole/l. against urine phosphorus also in mmole/l. The groups in the upper part of the graph received extra dietary calcium and the urine calcium is high while the phosphorus is low. The converse is seen on the lower part of the curve where the rats were given extra dietary phosphate. The shift of urinary calcium and phosphorus seems to behave with considerable mathematical precision. Moreover, simultaneous addition to the diet of both calcium and phosphate yields urine values back in the central bracketed segment of the curve where the results on normal diet are also found. So far as is known, the curve holds only for the particular circumstances of the experiment: the rat, the diet we use and calcium or phosphate added to the diet. If a new factor be introduced, the results may fall far outside the curve as shown in other experiments producing a metabolic distortion with vitamin D or desiccated thyroid. Finally, it may

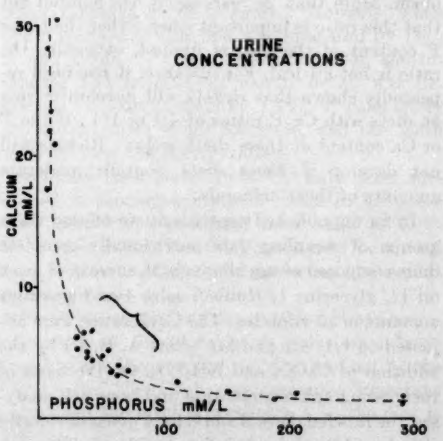


FIG. 7. Curve showing reciprocal shift in concentrations of calcium and phosphorus. See text for details.

be noted that foreign body stone formation occurs only in those rat groups where the urine values fell in the bracketed segment of the curve.

SUMMARY

When rats were fed extra calcium, the urine calcium rose to very high levels. Paradoxically, the formation of calcium phosphate stones was inhibited. Associated urine changes consisted of a profound drop in urine phosphorus and a sharp rise in urine citrate. In an ancillary experiment the rise in citrate was shown to be due to coincident alkalinization, not to the calcium per se. Other experiments were mentioned in which phosphate additions to the diet produced the reverse urine changes with a rise in phosphorus but a fall in calcium. The experiments illustrate the reciprocal relations existing between these 2 ions, presumably acting by alterations in gut absorption. This intimate interrelationship has received little attention in stone theory heretofore. Since the experimental conditions used in this investigation are highly artificial one dare not carry the conclusions over into the clinical situation without reservation. However, the observations do point up the error inherent in a common attitude toward stone disease in which urolithiasis is more or less equated with calcium, as if calcium were the only item of significance in the production of stones.

REFERENCES

1. Vermeulen, C. W., W. J. Grove, R. Goetz, H. D. Ragins and N. O. Correll. *J. Urol.* 64: 541, 1950.
2. Vermeulen, C. W. and R. Goetz. *J. Urol.* 72: 761, 1954.
3. Vermeulen, C. W. and R. Goetz. *J. Urol.* 72: 93, 1954.
4. Gill. W. B., B. Finlayson and C. W. Vermeulen. *J. Urol.* 82: 256, 1959.
5. Vermeulen, C. W., E. S. Lyon, W. B. Gill and W. H. Chapman. *J. Urol.* 82: 249, 1959.

DISCUSSION

Robert S. Harris

Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts

The important paper by Dr. Vermeulen suggests that we should emphasize the true meaning of the Ca:P ratio in the design of dietaries. Many believe that the Ca:P ratio must be held between 1:1 and 2:1 to avoid disturbances in mineral metabolism. More than 20 years ago it was pointed out that this ratio is important when either the Ca or P content of the diet is limited, otherwise the ratio is not critical. For instance, it has been repeatedly shown that rickets will develop in rats on diets with Ca:P ratios of 4:1 or 1:4 , if the P or Ca content of these diets is low. Rickets will not develop if these diets contain moderate amounts of these minerals.

In an unpublished experiment we offered three groups of weanling rats nutritionally complete diets composed of egg albumen 30, sucrose 48, corn oil 14, glycerine 1, Hubbell salts 4 and generous amounts of 16 vitamins. The Ca:P ratios were adjusted to 1:1, 4:1 and 8:1 (diets A, B, C) by the addition of $CaCO_3$ and KH_2PO_4. On the basis of roentgenograph examination and bone ash analyses, the rats fed diets B and C for 4 weeks developed rickets, while those fed diet A did not. This was expected.

Another group of weanling rats was fed diet D, which was identical to diet C except that the egg albumen was replaced by casein 30 and cystine 0.1. Though the Ca:P ratio of this diet was also 8:1, rickets was not observed (Fig. 1).

Casein is a phospho-protein. Therefore, the P content of diet D was 0.42% rather than 0.15% as in diet C. To determine whether the non-rachitic effect of the casein diet was due to its higher P content, an egg albumen diet similar to diet C was prepared by adding sufficient KH_2PO_4 and $CaCO_3$ to raise its P content to 0.42%, and to adjust its Ca:P ratio to 8:1. This diet (E) was non-rachitic (Fig. 1).

It should be emphasized that the production of rickets in rats depends on three conditions: *1*) a disturbed Ca:P ratio, *2*) absence of vitamin D and *3*) limited content of dietary P. We have failed to produce rickets in rats with diets including Ca:P ratios even as high as 20:1, when these diets contained more than 0.4% total P. I am of the opinion that the Ca:P ratio of diets is important when the Ca or P content are limited, but relatively unimportant if both these minerals are present in adequate amounts. Most diets in the United States contain adequate amounts of both Ca and P and, therefore, the Ca:P ratio may not be of critical importance. For 40 years nutritionists have con-

tended that the ideal Ca:P ratio in the nutrition of human beings lies between 1:1 and 2:1. The report by Dr. Vermeulen indicates that the conditions for kidney stone formation in rats are most favorable when the Ca:P ratio is approximately 1:1. Thus, the ratio most favorable to the uptake and deposition of Ca and P appears to be the ratio most favorable for stone formation. It is important to conduct careful studies on human subjects to establish this important point.

This is especially important because Dr. Vermeulen worked with rats, and rats differ from human beings in the metabolism of calcium. As a by-product of our studies of Ca^{45} metabolism during the past ten years, we have accumulated data on the routes of excretion of intravenously injected calcium in four species. The data in table 1 represent the averages from 80 children, 50 monkeys, 12 dogs and 40 rats. It will be noted that the healthy human being (adolescent or adult) excreted twice as much endogenous Ca^{45} in the urine as in the feces. The monkey also showed a urine:feces ratio of 2:1. But in the dog it was 1:10 and in the rat it was 1:22. On the basis of these data it may be questioned whether studies of the calcium metabolism of dogs and rats can be interpreted in terms of human beings. The interpretation of data from experiments with monkeys is less subject to error.

Our studies of monkeys with experimentally produced poliomyelitis paralysis, indicate that the paralysis causes a reduction of the urine:feces ratio of Ca^{45} excretion to 1.5:1, even though their excretion of calcium in the urine was abnormal (hypercalcuria). Therefore, the excretion of calcium via the feces was increased even more. The addition of sodium phytate to their diet caused a significant decrease in urinary calcium excretion. This type of effect has been observed by Vermeulen and Henneman *et al.* There is reason to believe that phytates may be used to treat hypercalcemia.

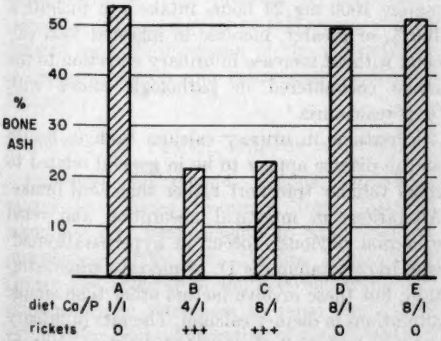


FIG. 1. Rachitogenic effects of Ca/P ratios.

TABLE 1*

Species	μc Ca^{45} Injected	% Dose Excreted (0-5 days)	Ratio Fecal/ Urinary Ca^{45} Excreted
Human, boys	0.7	8	1:2
Human, adult	3.4	20	1:2
Monkey, adult	1.7	9	1:2
Rat, adult	1.0	14	22:1
Dog, adult	1.5	42	10:1

* Reprinted from *Fed. Proc.* 15: No. 1, 1956.

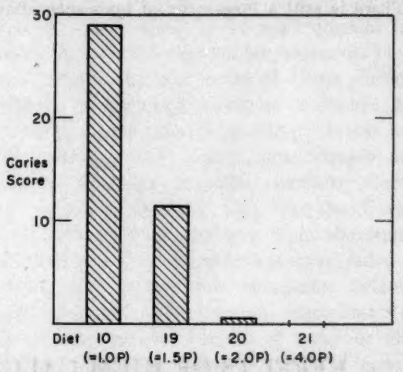


FIG. 2. Effect of HPO_3 in cariogenic diet on hamster caries.

The level of phosphorus in the diet may be more important in the control of dental caries than the Ca:P ratio. Our caries-producing diet (#10) is composed of whole corn meal 47, non-fat milk powder 20, sucrose 14, plastic cream 10, alfalfa 6, liver concentrate 2 and sodium chloride 1. It contains 0.37% P, and has a Ca:P ratio of 1:1. When fed to preweanling hamsters for 100 days it produced caries as shown in figure 2. However, when the P content of this diet was increased by 1½-, 2- and 4-fold by the addition of HPO_3, the incidence and severity of dental decay was decreased. A diet containing 1.48% P completely prevented caries in all hamsters, and the teeth were lustrous and pearly white.

We have tried many times, but have never succeeded in producing rampant caries in hamsters with diets which contained more than 0.5% P. With such a diet, the Ca:P ratio may be varied from 4:1 to 1:4 without influencing the dental caries picture.

Under the conditions of our experiment the development of dental caries in hamsters is governed by the P content of the diet, and not by the Ca:P ratio. The Ca:P ratio becomes important only when the level of available P in the diet is low.

During Dr. Harrison's able discussion of factors which influence the absorption of calcium, the pH of the intestinal tract was mentioned as one

important factor. The various calcium salts which are prescribed or are added to foods have an effect upon intestinal pH. Yet Bronner *et al.* (1) observed an equal uptake of Ca^{45} from calcium carbonate, calcium citrate, calcium gluconate, calcium lactate and from milk when these calcium sources were given at a level of 300 mg in the breakfast meals of 40 children. Apparently, healthy subjects respond to alkaline calcium salts by increasing the flow of gastric juice.

There is still a large area of ignorance about calcium metabolism. There is a minimum need for calcium in human nutrition. The level of calcium intake which appears optimum as measured by one criterion (i.e. bone formation) may not be optimum as measured by another criterion (i.e. renal stone formation). There is still no firm evidence that high calcium intakes are harmful.

REFERENCE

1. Bronner, F., J. R. Moor, R. S. Harris and C. E. Benda. *Fed. Proc.* 14: 428, 1955.

EFFECTS OF HIGH CALCIUM INTAKES ON URINE IN HUMAN BEINGS[1]

William H. Boyce and J. Stanton King, Jr.

Departments of Urology and Biochemistry, The Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina

Normal adult North Carolinians in calcium balance maintain average urinary excretion rates of 90 to 240 mg/24 hours over a range of intake from 150 to 2000 mg of calcium per day (1). Hodgkinson and Pyrah (2) reported similar values for 258 Britons. In both studies urinary calcium excretion was more constant for a given individual than from person to person within the group. The observation that alteration of calcium intake by variation of dietary milk products has no consistently proportionate effect on urinary excretion of calcium by normal subjects has been recorded by other investigators (3, 4). Howard, Parson and Bigham (5) found no appreciable alterations in the pattern of excretion of calcium by bed-ridden patients following increase of milk calcium intake from 200 to 2200 mg/24 hours. It seems evident that absorption of calcium in health is related more closely to need than to intake. Urinary calcium for subjects in balance therefore equals net intestinal absorption. This has been clearly presented by the Neumans (6) in their appropriate phrase 'the ion pumps' for intestinal absorption of calcium. One must conclude that the normal American who is in calcium balance on approximately 1000 mg/24 hours intake will tolerate a 100%, or greater, increase in ingested food calcium without increase in urinary excretion to the range encountered in pathologic states with 'hypercalcinuria.'

Variations in urinary calcium both in health and in disease appear to be in general related to renal tubular transport rather than oral intake. Alterations in intestinal absorption and renal excretion obviously occur in hyperparathyroidism, hypervitaminosis D, or phytate administration; but these involve factors other than simple alterations in dietary calcium. The rate of urinary calcium excretion is singularly independent of

[1] This work was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

total serum calcium concentration (3, 7). Chen and Neuman (8, 9) have demonstrated that the calcium content of the glomerular filtrate is approximately that of an ultrafiltrate of plasma. Complete reabsorption of the ionized calcium in the filtrate appeared to be accomplished by the tubular cells, suggesting the possibility that there may be no threshold for ionized calcium in health. The small but relatively constant amount of calcium in the urine was considered to be an un-ionized complex. Calcium clearance was increased by injection of the complexing agents, Versene® and citrate, or by administration of tubular metabolic inhibitors, phloridzin, dinitrophenol, or sodium azide. The amount of calcium in the urine is therefore related to the presence of unidentified complexing agents and to factors which influence the tubular transport mechanisms. These experiments do not preclude the possibility that tubular secretion makes some contribution to urinary calcium content. It is a common clinical observation that urinary calcium excretion of less than 90 mg/24 hours appears in patients with advanced renal tubular disease even when the diet contains one quart of milk per day and before glomerular filtration has been impaired to the point where azotemia becomes apparent.

It is not surprising, therefore, to find wide variations in excretion of urinary calcium associated with a variety of endocrinologic, metabolic, malignant, or other disease states. Even in the most severe forms of these diseases, the maximum urinary excretion of calcium approximates 1000 mg/24 hours and usually does not exceed 600 mg/24 hours. From a clinical point of view the only pathologic effect of hypercalcinuria is a possible relation to formation of urinary calculi. Hypercalcemia is much more commonly associated with disturbances of renal function than is hypercalcinuria. The remainder of this resumé will be devoted to a consideration of the interrelations of urinary calcium and calcigerous concrement formation.

INCIDENCE OF HUMAN URINARY CONCRETIONS

Historical Background. Bladder and renal calculi have been recovered from Egyptian mummies who were thought to have lived approximately 5000 B.C. Medical literature of Greece and India from the first millenium B.C. contains many references to renal and vesical calculi. The perineal operation of vesicolithotomy was probably performed several centuries B.C. by the Greeks and improved by the Arabian physicians during the seventh to the tenth centuries A.D. The problem of urinary calculi was ever present in the medical history of Europe, and both suprapubic and perineal operations for vesicolithotomy were reported from every European country during the Renaissance (10).

Geographic and Climatic Relationships. No area of the earth appears to be free of calculus formation, but certain 'stone belts' or regional areas in which calculi occur with unusual frequency have been recognized for centuries. These areas include the east coast of Great Britain, Holland, the east and southeast of France, the Balkan peninsula, the Volga valley, Arabia and Mesopotamia, lower Egypt, the Punjab and northern provinces of India, southern China, and northern Thailand (10). In the United States, the greatest incidence is in the southeastern states (11). All attempts have failed to produce any generalized correlation between stone areas and any common denominator of soil, climate, mineral content of water or diet. We have found no reported study of genetic factors in relation to calcigerous stone formation.

Seasonal and Temporal Variations. The incidence of calculi has varied from time to time in many countries, and is reported to have increased in Europe after every long war and to have decreased in periods of peace. The results of a survey of admissions to general hospitals in America over a five-year period revealed no decided year-to-year variation in incidence of urinary calculi (11).

There is a distinct seasonal variation in the incidence of small urinary calculi. These patients usually appear with one or more small calcium oxalate and/or phosphatic calculi in the ureter. The increased incidence begins in March, reaches a peak or plateau during August to September, then declines rapidly in October to a minimal incidence in December, January, and February. This pattern is well known to urologists and has been studied by Prince and Scardino (12), who related the incidence of calculi to the variations in mean temperature. Increased concentration of urinary solutes during hot weather does not appear to be a primary factor, since most of the stone-forming patients seen in this institution have 24-hour urinary volumes under everyday conditions of home life that are distinctly greater than the average volumes for normal individuals under similar circumstances. Furthermore, Mitchell and Hamilton (13) found a concentra-

tion of 2–7 mg of calcium per 100 ml of sweat, and estimated that the daily loss of calcium by this route could be as high as 149 mg under minimal sweating conditions. Persons subjected to high temperatures may lose as much as 4–14 mg of calcium per hour by this route (14).

We have observed no seasonal variation in urinary calcium excretion of stone-forming patients (1). However, there is a seasonal variation in the weight of total nondialyzable solids in the normal urine. This is closely parallel to the incidence of urinary calculi, an increase beginning in March and reaching maximal values in September. The change in urinary nondialyzable solids is primarily the result of alterations in the *UF-O* fraction (15). All patients who are actively forming calculi have marked increase in urinary nondialyzable solids. The presence of a stone does not necessarily influence the quantity of total nondialyzable urinary solids since we have observed many patients with quiescent renal calculi who had essentially normal values for the quantity of these solids per 24-hour urine volume. In no instance have we found these solids to be significantly less than normal in patients with calculi.

Race. No race is entirely free of calculi. The Negro race appears to have a very low incidence in America (11), although this may not be true in certain areas of Africa (16, 17). The variations in other races are less well established, and Jolly (10) suggested that, on the whole, calculi occurred in decreasing frequency among the Hindus, Arabs, southern Chinese and Anglo-Saxons.

Sex. All reported series of patients have indicated that calculi are more frequent in men than in women. During the seasonal increase in calculi the ratio is approximately 70 male–30 female, but during the remainder of the year the ratio in North Carolina is 54 male–46 female. Since these data refer to calculi of renal origin, the anatomical difference between the sexes are not considered to be important factors in these variations.

Age. In America the greatest number of patients with calculi are in the third and fourth decades of life. However, when compared with the survivors in the total population per decade, this age incidence is not statistically significant (fig. 1). In the United States, children are rarely victims of calculous disease, the over-all incidence of pediatric patients accounting for 0.3% of patients with calculi (11). In 1928 Hager and Magath reported 2195 cases of vesical calculous

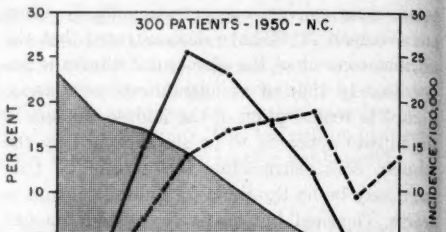


FIG. 1. The peak age incidence (25%) of 300 consecutive new hospital admissions for renal calculi in 1950 was in the third decade of life. When the number of patients in each decade was extrapolated to survivors of comparable age in the North Carolina population (1950 census), there appeared to be a progressive increase in the incidence of initial attacks of renal calculi from age 19 to age 60.

disease, 1.63% of which occurred in children under 15 years of age (18). In 1951 Lattimer and Hubbard found no cases of urinary calculi among 21,835 admissions of children less than 13 years of age to Babies Hospital in New York City (19). There has not been a single patient with 'idiopathic' urinary calculi admitted to the Pediatric Service of the North Carolina Baptist Hospital in the past 17 years. In each of the pediatric patients with calculi there has been a clearly established diagnosis of systemic disease to account for the calculus formation. These included congenital systemic acidosis (renal tubular disease with inability to acidify the urine), 'oxalosis,' classical cystinuria, congenital exstrophy of the bladder, or other anomalies of the urinary system.

In Asia the incidence of calculi is much greater in children. Thomson (20) reported that 25% of 2962 cases of vesical calculi seen in Canton, China, were in children under ten years of age. A survey of calculi in Thailand in 1953 indicated that more than two-thirds of all urinary calculi occurred in children, and the majority of these were limited to the urinary bladder (21). The recurrence of these vesical calculi following surgical removal was reported to be practically unknown.

Occurrence of Calculi in Animals. Every type of crystalline calculus found in man has been

reported in other mammalian forms. Both herbivorous and carnivorous animals, including horses, cattle, sheep, dogs, cats, minks, rabbits and rats, form urinary calculi. Siliceous calculi occur spontaneously in cattle (22), xanthine calculi occur in sheep (23), cystine calculi in minks and dogs (24), and uric acid calculi in the Dalmatian coach hound (25). The experimental production of urinary calculi in laboratory animals has usually required such extreme alterations of urinary structure and metabolism that Keyser was prompted to state in 1943: "No stone without preceding trauma to urinary epithelium seems an axiom of the present day" (26). The results of such experiments may not be unequivocally interpreted in terms of a single metabolic pathway, such as calcium transport. One must certainly have reservations in drawing correlations between experimental and human calculus formation.

COMPOSITION AND STRUCTURE OF HUMAN URINARY CONCRETIONS

All urinary calculi are composed of an organic matrix and interspersed crystals. The matrix is always present from center to surface and is of essentially the same composition in all calculi. The crystals are exceedingly variable in composition, structure and distribution, but are never found as a crystalline mass without an organic matrix.

Crystalline Composition. The crystals are predominantly calcigerous in 90–94% of all calculi. Hydroxy apatite is the most common crystal, being readily detectable in 75% of calculi (27). The universal presence of calcium and phosphorus in the matrix of calculi suggests that this crystal is probably present to some extent in all urinary concretions. Uric acid and cystine are the predominant crystals in approximately 4 and 1% of calculi, respectively. Calcium oxalate is present in approximately 50% of calculi as the monohydrate or dihydrate, and magnesium ammonium phosphate is present in approximately 30%. By ordinary methods of x-ray diffraction, not more than 10% of 600 calculi studied by Lagergren (27) were classified as 'pure', i.e. contained a single crystal type.

Matrix Composition. The organic matrix is a mucoid containing approximately 63% protides, 13% glucides, 4% hexosamine, 11% 'bound' water and 8% bound inorganic ions or radicals (28). The protein moiety contains at least 18 common amino acids and has not been separable intact from the carbohydrate moiety by acid or alkaline hydrolysis. The carbohydrate moiety contains galactose, mannose, glucose, rhamnose, fucose, hexosamine and deoxypentose (29).

Structure of Calculi. Microscopically the organic matric appears in both fibrous and amorphous forms (30), which appear to correspond respectively to buffer-insoluble and buffer-soluble portions of matrix. The latter are quite similar in their over-all composition (28), suggesting that the difference in microscopic appearance reflects a physical rather than a constitutional difference. The fibrils are arranged in parallel orientation of the long axes to form laminations. Variable quantities of amorphous matrix and fine fibrils appear in the interlaminar spaces. We have never seen a calculus without a matrix, but, occasionally, calculi are composed almost entirely of matrix. These 'matrix calculi' have the same structural characteristics as the fully crystallized calculi (31). They are radiolucent by ordinary methods of roentgenography, and may be sectioned with the microtome without prior decrystallization.

Crystal matrix interrelations have been studied by microradiography (27, 32), conventional microscopy of decrystallized calculi (33) and petrographic microscopy. The conventional calculus usually appears to be uniformly crystalline on gross examination of the cut surface. Microradiography has demonstrated definite structural relations of crystals to matrix with marked variations in the number of crystals per unit volume of calculus. The hydroxy apatite crystals are microscopically always aligned with the fibrous matrix; whether this reflects axial orientation of the individual crystals is unknown. The magnesium ammonium phosphate crystals appear to have no microscopic orientation to the fibrous matrix. Other crystalline types appear to have variable degrees of predilection for the fibrous matrix.

Origin of Calculous Matrix. The origin of calculous matrix remains unknown. Immunochemical studies in our institution (manuscript in preparation by P. Baram) have demonstrated the presence of a soluble component of calculous matrix which is present in normal human serum but absent from normal urinary nondialyzable solids. This unidentified material is not detectable in the serum of calculous disease patients but is present in their nondialyzable urinary solids. The implication of renal 'permeability' to a component of matrix is obvious, but much

additional investigation must be completed before conclusions are warranted.

The physical properties and/or chemical analysis of collagen and osseomucoid of human bone matrix and of matrix from human gall stones has demonstrated that these substances are distinctly different from urinary calculous matrix (28). The *R-1* fraction of normal human urine (15) is more comparable to the insoluble portion of urinary calculous matrix than any other substance yet isolated from the total nondialyzable solids of urine or from human blood plasma. The *R-1* 'uromucoid' of normal urine has more glucides than stone matrix and inconstantly contains sialic acid. Normal *R-1* urinary mucoid shows little tendency to molecular aggregation, although thixotropic gel formation can be induced in cold preparations by gradual shift of pH from 8.6 to 4.5.

It has been suggested that enzymatic or other partial degradation of the carbohydrate moiety of *R-1* urinary mucoid may result in loss of sialic acid and some of the other terminal groups with reduction of glucide and occasionally of hexosamine content (31). These changes may influence both the ion-binding and molecular aggregation of *R-1* mucoid (34). Data on 'bound' inorganic ash, with relative calcium and phosphorus content, of the three major fractions of urinary total nondialyzable solids (TNDS) are presented in figure 2. The total quantity of nondialyzable solids is invariably increased in patients who are actively forming calculi, and the total 'bound' calcium is correspondingly in-

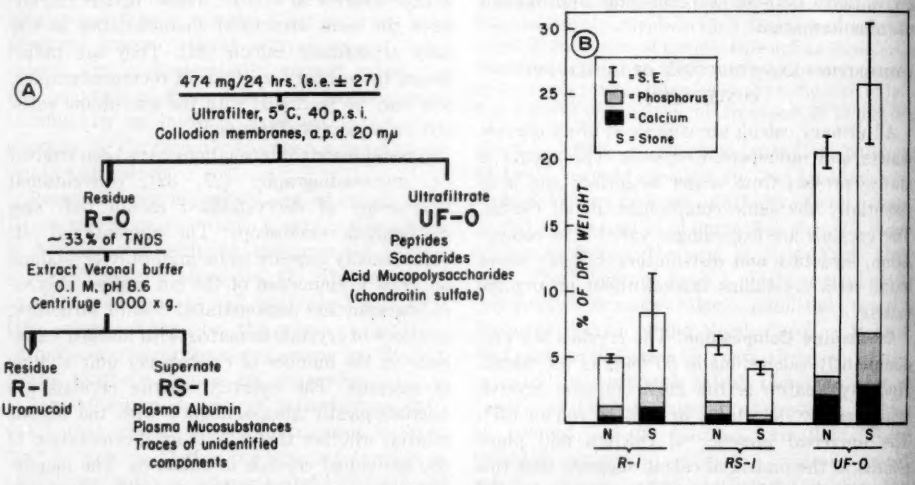


FIG. 2. *A:* Outline of method for separation of total nondialyzable solids of urine into three major fractions. Normal *R-1* fraction accounts for approximately 20% of the total and is composed of mucoid(s) which are probably of renal tubular or transitional epithelial origin. The *RS-1* fraction accounts for approximately 13% of the total normal solids and contains all of the serum albumin and those plasma mucosubstances which are identifiable in urine. These mucosubstances include components which have electrophoretic mobilities at pH 8.6 that are identical with those of all the recognized serum globulin components; however, their isoelectric points are less than that of serum albumin, and hence they are distinctly different from the majority of the serum globulins. The *UF-O* fraction of normal urine accounts for approximately 67% of the weight and contains practically all of the hexuronic acid of the total solids. The glucide content of the *UF-O* is significantly greater than that of the other fractions. Patients with active renal calculus formation exhibit considerable variation in the 24-hr. excretion rates of all of these three fractions—a variation which is apparently the result of degrees of injury to the nephrons and the urinary system from infection and obstruction to urine flow.

B: Inorganic ash content in terms of per cent of dry weight of fractions of total nondialyzable solids recovered from the urine of 12 normal subjects (N) and 10 patients (S) who were actively forming renal calculi. The lyophilized dry weights of the solids in each fraction, expressed as mg/24 hr. were:

	R-1		RS-1		UF-O	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Normal (N)	90	11	59	7	264	29
Calculus (S)	293	174	407	112	785	352

creased. There is often a marked reduction both in TNDS and urinary calcium immediately following major surgical procedures. However, there is no consistent correlation between the quantity of TNDS and the total urinary calcium, either between individuals within the group or from time to time for the same person. The standard error of the mean (fig. 2) may be more fully appreciated when one is acquainted with the extreme range of variation in TNDS which occurs from time to time in the individuals with growing calculi. The minimum and maximum quantities in mg/24 hours which we have observed in these stone patients are respectively 113–595 for *R-1* fraction, 303–604 for *RS-1* fraction and 142–2050 for *UF-O* fraction. The *RS-1* fraction of active calculous disease contains relatively less alpha-1 mucosubstances than does average normal *RS-1*, and this may account for the reduced capacity for calcium binding by the *RS-1* of calculous disease urine. The increased calcium binding capacity of *R-1* of calculous urine is in accord with the concept that structural alterations of a portion of this material may precede stone matrix formation.

The basic mechanism of calculus formation may be summarized in the following interpretative comments.

The *R-1* urinary mucoid is probably of urinary epithelial origin, either from the renal tubules or the transitional epithelium. In the normal state, there is some binding of calcium and phosphorus by the molecules, but little tendency to molecular aggregation.

Partial degradation of the carbohydrate prosthetic groups of *R-1* uromucoid may enhance the ion-binding capacity and permit the observed parallel orientation of the molecules to form fibers. Molecular orientation occurring in a gel of *R-1* uromucoid may produce the structural characteristics of the calculus matrix. The resultant laminations with their spatially arranged calcium, phosphate and hydroxyl groups act as templates or epitactic stimuli to apatite crystal formation. As the laminations condense into semipermeable membranes, the interlaminar

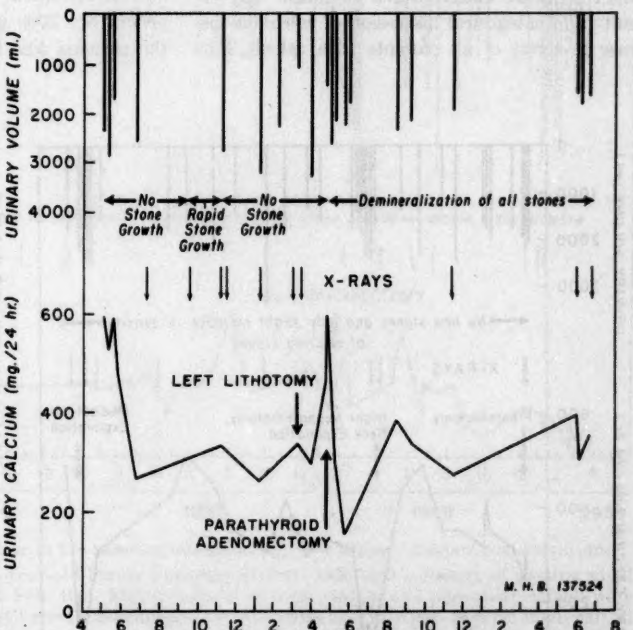


FIG. 3. Calculus formation and reabsorption with essentially the same rate of urinary calcium excretion. *M. H. B.*, NCB #137524, a 41-year-old male was first seen in April 1956 with a history of passing small calculi from either kidney every 1–3 months for 12 yr., and multiple small calculi were present in all the minor calyces of both kidneys. Urinary calcium excretion was consistently above 527 mg/24 hr. on an estimated intake of 800–1000 mg/day for 1 month. Patient placed on 150 mg Ca intake plus sodium phytate with reduction of urinary calcium to approximately 300 mg/24 hr. X-ray on Nov. 6, 1956, revealed a large calculus in the left kidney, which had formed since the previous x-ray, Sept. 19, 1956. No further growth of this calculus occurred during 4 months of observation, and it was removed on Mar. 1, 1957. Following removal of a parathyroid adenoma (May 1957) the urinary calcium excretion stabilized at approximately 300 mg/24 hr. Within 12 months all concretions in each kidney were completely reabsorbed and no new ones have formed to date. Note reduction in urine volume following parathyroidectomy. The mean concentration of calcium per unit volume of urine was greater during the period of stone reabsorption than during stone formation.

spaces provide a favorable environment for crystallization. The composition of these secondary crystals is largely determined by the ambient conditions prevailing in the urine, and hence is subject to considerable variation.

The organic matrix appears to be prerequisite to calculus formation, while crystal deposition is a secondary phenomenon. The matrix in its architectonic capacity is largely responsible for the size, shape, concentric laminations, radial striations, color and, in a sense, the crystal composition of calculi (31).

THE INFLUENCE OF URINARY CALCIUM ON URINARY CALCULUS FORMATION

Hypercalcinuria with Calculus Formation. The biochemical disorders responsible for urinary calculus formation may be associated with any one of a number of endocrinologic, metabolic, cancerous, traumatic, degenerative, or infectious diseases, as well as diseases localized to the urinary system (35). The presence of a calculus must therefore be regarded as a symptom rather than a disease entity. Much confusion may result from categorical discussion of calculous disease in terms of all patients with calculi. Furthermore, the growth of calculi in the human is usually sporadic, and seldom, if ever, sustained at a constant rate for any prolonged period of time. Spontaneous or therapeutically induced reversal of growth is extremely rare, having been observed by us in only one patient, following removal of a parathyroid adenoma (fig. 3).

It is estimated that in 1952 a minimum of 1.4 persons per 1000 population in North Carolina required hospitalization because of urinary calculi. Of this group, fully 80% exhibit only one or two episodes of calculus formation in their entire lives. These patients have no evidence of systemic or urinary disease. Unless urinary infection or other complications supervene, neither are they problems in management nor do they have recurrent calculi. Urinary calcium excretion rates on self-selected diets are no greater in this group than in normal persons from the same population (1).

The remaining 20% of 'new' patients with calculi are diagnostic and therapeutic problems with bilateral or recurrent calculus formation. By extrapolation, these patients represent one person per 3000 general population. These are the patients who find their way to the medical

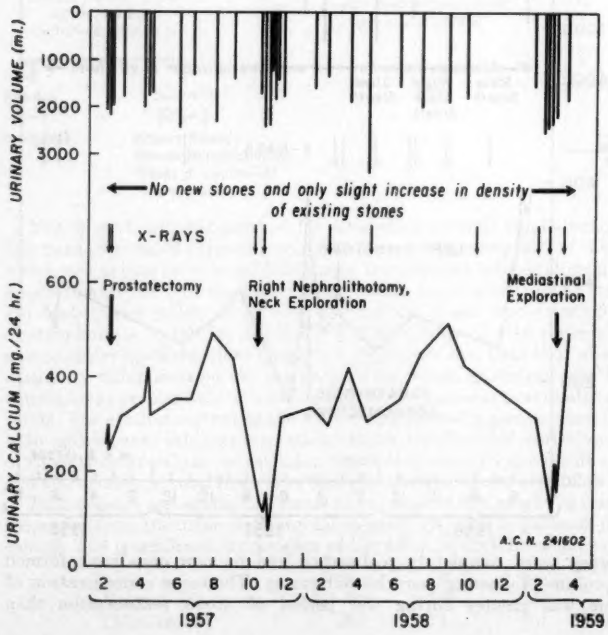


FIG. 4. Hypercalcinuria without calculus formation in a patient who had previously demonstrated ability to form calculi. *A. C. N.*, NCB #241-602, a 48-year-old male with bilateral multiple renal calculi and massive calcification of the prostate. Prostatic calculi removed on FEB. 6, 1957. All stones removed from right kidney on Oct. 21, 1957. All parathyroid glands identified and 3 removed (histologically normal) on Oct. 30, 1957. Exploration of mediastinum in Feb. 1959 revealed no parathyroid adenoma. With the exception of low urinary calcium excretion following surgery, there has been a consistently elevated urinary calcium. No new calculi have developed in either kidney, and only slight increase in size of existing left renal calculi has occurred in two years of observation.

centers and who form the subjects for many of the reported series of 'stone forming patients.' Approximately 80% of this group will have hypercalcinuria at any given time of study. In our own series, the individual mean urinary calcium excretion of 60 such patients was between 325 and 350 mg/24 hours during periods when they were performing their usual physical activity, and were on self-selected diets containing an estimated intake of 1000 mg (±200) of calcium daily.

Approximately one in five of these 'problem patients' will have one of the endocrinologic, metabolic, malignant, or other diseases recognized as etiologic factors in renal calculus formation (fig. 3). Again by extrapolation, these patients represent approximately one person per 15,000 general population. The hypercalcinuria in these patients remains 'idiopathic' in fully 80% of the group, but abnormally high ingestion of calcium is not an etiologic factor in North Carolina, if one excludes the occasional patient with peptic ulcer and concomitant renal calculi.

Hypercalcinuria Without Calculus Formation (fig. 4). Although the above patients with rapidly recurrent and/or bilateral calculi commonly have hypercalcinuria, we have been unable to form any consistent correlation between the rapidity of new calculus formation or rate of calculus growth and the level of urinary calcium in the individual patients (fig. 4). That is to say, those patients with rapidly growing large calculi usually have the same range and mean calcium excretion rates as do those who form only small calculi.

Thirty-eight per cent of our apparently normal subjects were found to have urinary calcium excretion rates of between 200 and 275 mg/24 hours (1). There are many patients in whom hypercalcinuria much greater than that of the problem stone patient may persist for months or years without urinary calculus formation. These in-

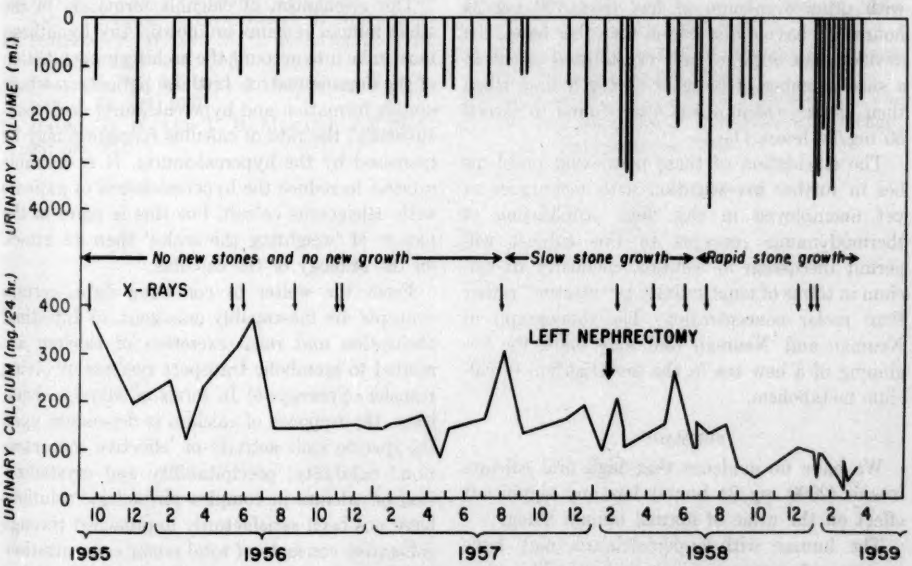


FIG. 5. Maximal stone formation in the presence of exceedingly low urinary calcium concentration.

L. McI., NCB #067149, a 44-year-old female first seen in Sept. 1955 with a history of passing small calculi from either kidney since Feb. 1941. Multiple small calcium oxalate and phosphate calculi were present in the left kidney, a small intrarenal calcification was noted on the right. Several small calculi were recovered from the left kidney by spontaneous passage or ureterolithotomy during 1956, but no new calculi formed in the right kidney. Left nephrectomy was done Feb. 18, 1958, because of multiple renal abscesses and septicemia (*B. proteus*). Within 3 months after this procedure, slowly progressive increase in the right intrarenal calcification was noted. Subsequently a large branched calculus completely filled the right pelvio-calyceal system within a period of 6 wk. This kidney had not previously formed a calculus in at least 7 yr. Note the maximal urine volume and minimal urinary calcium during the phase of rapid stone growth.

clude surgical hypoparathyroidism under therapy, 30–55% of patients with hyperparathyroidism and, indeed, a high proportion of all patients with endocrinologic or metabolic diseases of the type considered to be potential stone formers.

A particularly interesting group of patients are those with disorders of thyroid metabolism. The patient with thyrotoxicosis has a normal concentration of serum calcium, but urinary calcium excretion rates are commonly in the range of 300–500 mg/24 hours. The cretin, on the other hand, has a urinary calcium excretion rate of less than 100 mg/24 hours in spite of an elevated serum calcium. We have observed one case of spontaneous calculus formation in a cretin, but never have we seen a patient with hyperthyroidism and urinary calculi.

Hypocalcinuria with Calculus Formation (fig. 5). The minimum urinary calcium content required for calcigerous stone formation is unknown. Most 'matrix calculi' develop in patients with urinary calcium of less than 100 mg/24 hours. We have observed, on the other hand, the development of large, fully crystallized calculi in a small number of patients during a time when their urinary calcium was never found to exceed 50 mg/24 hours (1).

The elucidation of these perplexing problems lies in further investigation with techniques as yet unemployed in this field. Application of thermodynamic concepts to this subject will permit discussion of solution chemistry of calcium in terms of ionic activity or 'effective' rather than molar concentration. The monograph of Neuman and Neuman (36) may mark the beginning of a new era in the investigation of calcium metabolism.

SUMMARY

We have no evidence that high oral calcium intake (2000 mg/24 hours) has any significant effect on the urine of normal human beings.

The human with hypercalcinuria may have any one of numerous disease states. The renal response of such an individual to either oral calcium load or restriction is correspondingly unpredictable. The hypercalcinuria tends to remain fairly constant for each individual over many months and to be surprisingly independent of the calcium intake. In general, reduction in calcium intake to 150 mg/24 hours plus maximal doses of sodium phytate (12 gm/24 hours) is necessary to effect a significant reduction in the urinary calcium of such patients. Even then, the individual tends to stabilize at some level of calcium excretion which may be well above the normal range, and in some instances continues to form calcigerous stones even with a reduced urinary calcium content. There is no good evidence that high oral calcium intake has any direct cause and effect relationship to the initiation of renal calculus formation.

Urinary calculi are among the most ancient of recorded diseases. The greatest incidence has been recorded in times (post wars) during which, and in areas (China, India, Thailand) where, diets were substandard by modern criteria. Even in animals the spontaneous formation of calculi has been related to environments which probably represent alterations of metabolism other than calcium intake. The occurrence of 'idiopathic' calculi is practically unknown in American children, whose diets are enriched by all the minerals, vitamins and supplements of modern food processing.

The mechanism of calculus formation in the adult human remains unknown. Any hypothesis must take into account the architectonic function of the organic matrix. In those patients in whom matrix formation and hypercalcinuria occur concurrently, the rate of calculus formation may be increased by the hypercalcinuria. It is desirable a priori to reduce the hypercalcinuria of patients with calcigerous calculi, but this is more in the nature of 'weighting the scales' than an attack on the etiology of the calculus.

From the welter of confusing data, certain concepts are inescapably emergent. *a*) Intestinal absorption and renal excretion of calcium are related to metabolic transport systems involving transfer of energy. *b*) In terms of solution chemistry, the response of calcium is dependent upon the specific ionic activity or 'effective concentration.' Solubility, precipitability and crystallization of calcium in complex physiologic solutions have not been satisfactorily approached through colligative concepts of total molar concentration. *c*) The intact mammal is well equipped to deal with an exceedingly wide range of dietary calcium intake in the form of naturally occurring foods without the development of pathologic conditions unless there are also related alterations of calcium transport systems.

REFERENCES

1. Boyce, W. H., F. K. Garvey and C. Govan. *J.A.M.A.* 166: 1577, 1958.

2. HODGKINSON, A. AND L. N. PYRAH. *Brit. J. Surg.* 46: 10, 1958.
3. HOWARD, J. E. *J. Bone Jt. Surg.* 33-A: 801, 1951.
4. NICOLAYSEN, R., N. EEG-LARSEN AND O. J. MALM. *Physiol. Rev.* 33: 424, 1953.
5. HOWARD, J. E., W. PARSON AND R. S. BIGHAM, JR. *Bull. Johns Hopkins Hosp.* 77: 291, 1945.
6. NEUMAN, W. F. AND M. W. NEUMAN. *Am. J. Med.* 22: 123, 1957.
7. DENT, C. E. In: *Ciba Foundation Symposium on the Kidney*, edited by A. A. G. Lewis and G. E. W. Wolstenholme. Boston: Little, 1954, p. 242.
8. CHEN, P. S. *Fed. Proc.* 12: 310, 1953.
9. CHEN, P. S. AND W. F. NEUMAN. *Am. J. Physiol.* 180: 623, 1955.
10. JOLLY, J. S. In: *Stone and Calculous Disease in the Urinary Organs*. St. Louis: Mosby, 1931.
11. BOYCE, W. H., F. K. GARVEY AND H. E. STRAWCUTTER. *J. A. M. A.* 161: 1437, 1956.
12. PRINCE, C. L., P. L. SCARDINO AND C. T. WOLAN. *J. Urol.* 75: 209, 1956.
13. MITCHELL, H. H. AND T. S. HAMILTON. *J. Biol. Chem.* 178: 345, 1949.
14. JOHNSTON, F. A., J. T. MCMILLAN AND E. R. EVANS. *J. Nutrition* 42: 285, 1950.
15. BOYCE, W. H., J. S. KING, JR., J. M. LITTLE AND C. ARTOM. *J. Clin. Invest.* 37: 1658, 1958.
16. HORNER, W. H. AND M. C. HORNER. *J. Urol.* 68: 929, 1952.
17. POLITZER, W. M. AND A. BEUCHAT. *S. Afr. med. J.* 31: 311, 1957.
18. HAGER, B. H. AND T. B. MAGATH. *J.A.M.A.* 90: 266, 1928.
19. LATTIMER, J. K. AND M. HUBBARD. *J. Urol.* 66: 289, 1951.
20. THOMSON, J. O. *Surg. Gynec. & Obst.* 32: 44, 1921.
21. PASSMORE, R. *Lancet* 1: 638, 1953.
22. KEELER, R. F. AND K. F. SWINGLE. *Am. J. Vet. Res.* 20: 249, 1959.
23. ESTERFIELD, T. A. AND J. A. BRUCE. *Vet. J.* 86: 251, 1930.
24. OLDFIELD, J. E., P. H. ALLEN AND J. ADAIR. *Proc. Soc. Exper. Biol. & Med.* 91: 560, 1956.
25. HIGGINS, C. C. In: *Renal Lithiasis*. Springfield: Thomas, 1943.
26. KEYSER, L. D. *J. Urol.* 50: 169, 1943.
27. LAGERGREN, C. *Acta Radiol.* 133: 1, 1956.
28. KING, J. S., JR. AND W. H. BOYCE. *Arch. Biochem. and Biophys.* 82: 455, 1959.
29. KING, J. S., JR. AND W. H. BOYCE. *Proc. Soc. Exper. Biol. & Med.* 95: 183, 1957.
30. BOYCE, W. H. AND N. M. SULKIN. *J. Clin. Invest.* 35: 1067, 1956.
31. BOYCE, W. H. AND J. S. KING, JR. *J. Urol.* 81: 351, 1959.
32. BOYCE, W. H., C. S. POOLE, I. MESCHAN AND J. S. KING, JR. *Acta Radiol.* 50: 543, 1958.
33. BOYCE, W. H. AND F. K. GARVEY. *J. Urol.* 76: 213, 1956.
34. BOYCE, W. H., F. K. GARVEY AND C. M. NORFLEET. *J. Urol.* 72: 1019, 1954.
35. GARVEY, F. K. AND W. H. BOYCE. *J. Internat. Coll. Surgeons* 25: 310, 1956.
36. NEUMAN, W. F. AND M. W. NEUMAN. In: *The Chemical Dynamics of Bone Mineral*. Chicago: Univ. Chicago Press, 1958.

EFFECTS OF HIGH CALCIUM INTAKES ON BONES, BLOOD AND SOFT TISSUE; RELATIONSHIP OF CALCIUM INTAKE TO BALANCE IN OSTEOPOROSIS

G. DONALD WHEDON

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

CONCERN HAS RECENTLY been expressed over the possible ill effects of the administration of high dietary calcium intakes. Evidence for such effects should be examined, but in the presence of concern it is equally important to review data bearing on the possible benefits of high calcium intakes. Particular attention will be given in this report to the blood and soft tissues for effects which might predispose to untoward sequelae, and to the bones for indications of possible value.

BLOOD

First, with respect to the influence of high dietary calcium intakes on the level of blood calcium, no relationship seems demonstrable. Theoretically, at least temporary small increases in blood calcium must occur shortly after ingestion, but with calcium from ordinary dietary sources such changes are lost in the insensitivity of current analytical methods (1, 2). Apparently in the normal animal or human the mechanisms for maintaining blood calcium levels within a relatively narrow normal range deal readily with the amounts of calcium presented from the intestine. Even in the acute situation, calcium contained in ordinary foods is absorbed so slowly that blood calcium levels are not deranged to a detectable degree (2). Greenberg and Gunther (3) noted no change following the rapid ingestion of one liter of regular sweet milk. When an equal quantity of acid milk was taken, serum calcium rose 1 mg% in 2 hours and returned to normal in 4 hours. These same investigators tested the effects of various calcium salts given orally and found rises in blood calcium proportional to the dose; 1 gm of calcium, as calcium, being necessary to obtain a rise of approximately 1 mg% and 2 gm for 1.5 mg%. For this latter increase 7 gm of calcium chloride had to be given, 8 gm of the acetate, 11 of the lactate and 22 of the gluconate. With regard to ingestion of high calcium intakes over long periods of time, Malm's recent monograph (4) does not report hypercalcemia in males after several months of so-called high intakes of 900 to 1000 mg. In our patients with osteoporosis, (data for which will be presented more fully a little later) several weeks on daily intakes of calcium up to 1600 and 2400 mg produced no change in blood calcium levels. In the recently described syndrome in infants, called 'idiopathic hypercalcemia' (5), an influence of dietary calcium is currently regarded as improbable in view of the high concentration of the disease geographically and its occurrence among breast-fed infants.

The data just mentioned all relate to the lack of effect of dietary calcium alone. However, when agents are given which are known to influence blood calcium by themselves, there is a suggestion that high calcium intakes may accentuate these elevations. The hypercalcemic agents are parathyroid hormone (6), vitamin D in large amounts (1, 2) and alkalis.

SOFT TISSUE

With regard to increases in concentration of calcium or deposition in soft tissues the evidence for effect from high calcium intakes alone is as negative as it is with blood concentrations. Hansard and Plumlee (7) gave 250 growing rats 4 different levels of calcium intake over a 100-fold range, from 0.01% (0.8 mg) to 1.0% (102 mg). The dietary calcium intake level was not reflected in the analytical values for the soft tissues.

Metastatic calcification is relatively uncommon in human pathology but occurs when there is a well established hypercalcemia in association with increased bone resorption as found in chronic renal disease, osteolytic carcinoma, multiple myeloma and hyperparathyroidism (8). Tissue deposits of calcium have been produced experimentally by alkali, vitamin D and parathormone, but not apparently by high calcium alone (8, 9); the latter result is understandable since it is so difficult to raise the blood level of calcium by its oral administration. Since tissue calcification is also noted with hypocalcemia, the importance is placed on local factors such as alkalinity and local cell destruction or devitalization.

In the rare human disorder, *calcinosis universalis*, a metabolic balance study by Bauer, Marble and Bennett (10) showed low urinary and fecal calcium excretion regardless of the level of calcium intake; it was impossible to determine whether the calcium stored on high intake went particularly to bone or to soft tissue but since it could be expected to go to the latter, high calcium intakes are interdicted in this rare clinical state.

During the past decade much attention has been accorded to an unusual combination of signs called the 'milk-alkali syndrome' (11). Deposits of calcium in the membranes of the eye, and in various other tissues in association with hypercalcemia and renal damage, have been noted in certain peptic ulcer patients who for many months had taken large quantities of absorbable alkaline powders and more than one quart of milk per day. Kidney damage has been present in nearly all of these patients, but despite this association it is not certain that this is essential for development of the syndrome. Since physicians have promptly ordered both reduction in milk and shift in alkali to a non-absorbable type when the diagnosis was made, which agent is the more significant etiologically cannot be determined. The failure of milk alone to produce even temporary hypercalcemia, and the apparent key role of alkali in experimental tissue calcification, points to the essentiality of alkali for this uncommon untoward event associated with high calcium intake. It seems safe to continue to recommend milk for the peptic ulcer patient as long as non-absorbable alkaline gels are prescribed for intestinal antacid control.

BONES

Now we must deal with the bones. As is said in Boris Pasternak's *Doktor Zhivago* (12). "It's the bones that matter, the soft part doesn't matter at all."

High calcium intakes are prescribed principally because it is anticipated that there will result either increased growth or increased rigidity of the bones. Those who are unenthusiastic about calcium (13) feel so chiefly because they believe no advantage of this sort (or any other) has been demonstrated. Since spelunkers and jockeys see no merit in size, it may be that the key advantage which needs to be demonstrated is improved bone strength. The particular study on this point is that of Bell, Cuthbertson and Orr (14) who found, in rats fed calcium over a 5-fold range from 0.07% to 0.36% of the diet, a proportional relationship between calcium intake and bending and twisting strengths of the femora, as well as thickness of the femoral cortices and femoral weight, and calcium content. As a frame of reference, in the human, a calcium intake of 1.0 gm in a diet of 2500 calories would be approximately 0.2% calcium on a dry weight basis. No comparable experiment has been done in the human, but in this species it is evident to orthopedists that likelihood of fracture is proportional not merely to degree of trauma but also at any age to diminished bone density as seen by x-ray regardless of the cause of diminished calcium content. If this be valid, then increased importance needs to be focused on the difficult question of determining whether increased amounts of intake calcium will alone influence the density of bone.

In animals, various experiments have indicated increases in bone mass resulting from high calcium intake. Rats on high calcium intakes for many months have shown a higher bone calcium content than rats on a lower, although theoretically sufficient, calcium intake (15, 16). Stepwise reduction of calcium intake in adult rats resulted in loss of trabecular bone (17). Adult cats maintained for 80 days or more on a high, then a low calcium intake and vice versa, showed bony trabeculae which were diminished on low calcium and thickened on the high calcium intake (18); a co-author of the latter paper was Albright. Three years ago at these meetings a paper (19) was presented showing radiographic bone density changes in rat femora resulting from alterations both up and down in dietary calcium.

In human beings, data of this type are limited and less specific. Indian school children given calcium lactate supplements gained in both height and weight over their controls (20). Just recently a study (21) of customary calcium intakes showed 12% higher radiographic phalangeal bone density in individuals accustomed to intakes of more than 1250 mg than in those taking between 350 and 750 mg.

The remaining human data are based on metabolic balance studies. The premise on which such studies are thought to bear on this question is that since 99% of the calcium in the body is housed in the skeleton (22) and since, as I have earlier indicated, increased calcium intakes do not result in measurable increases in blood or soft tissue content, any calcium storage over an extended period of time must be taking place in bone. Balance studies of only a week or two reflect the prior level of calcium intake, suggesting

alteration in the size of the exchangeable calcium pool (relatively very small in comparison to the mass of skeletal calcium), but if positive balances continue over many weeks or months the mineral has nowhere to go except to bone. Whether the studies involving intake level changes thus far carried out have been long enough may be debatable, but a positive correlation between calcium intake and balance in individuals without disease has been shown by several investigators, including Leichsenring (23), Steggerda and Mitchell (24), Ohlson (25), Roberts (26) and Spencer (27). The possibility that older females may require higher levels of calcium for equilibrium than males, is suggested by some of these studies (26).

In normal adult males the longest continued calcium balance studies seem to be those recently published by Malm (4). Thirty-nine men over a wide age range were studied for an average of 7 months on a 'high' intake averaging 937 mg/day. The data showed a mean positive balance of 78 ± 12 mg/day. This represents a mean total Ca gain of 17 gm. Many of these men were also in a group studied at low calcium intake (mean 459 mg/day) for which the balance averaged −33 mg/day. On low intake the men showed adaptation, the balances generally becoming less negative as the months on low intake went by; on high intake, however, these normal males tended to maintain the same level of positive balance.

SIGNIFICANCE OF CALCIUM INTAKE IN OSTEOPOROSIS

The question of whether calcium can be stored in appreciable amounts and in continuing fashion by high calcium intakes is of considerable interest, at least to a clinical investigator, if it could be answered affirmatively in demineralized clinical states. Osteomalacia, which is characterized by a diminished concentration of mineral per unit of bone mass, is generally considered to be less prevalent in this country than its cousin, osteoporosis. Osteoporosis is widely thought to involve no defect in calcium metabolism, but rather to owe its diminution in bone mass to failure of osteoid or matrix formation in association most frequently with either immobilization, an absolute decrease in gonadal steroid elaboration or a relative decrease in the latter in proportion to adrenal cortical steroid production. Nutritional etiology of osteoporosis is only thought of in relation to deficiency either of vitamin C or of protein intake. Despite the plausibility and apparent adequacy of this etiologic classification, however, the possibility has occasionally been raised recently that the mineral, calcium, has some direct relationship, either pathogenetic or therapeutic, to this condition in which bones manifestly contain less calcium than normal. The most subtle way of introducing this subversive (or antique?) thought is to suggest that calcium lack may be a contributory or additional factor which could accentuate the demineralizing (or hypo-mineralizing) process initiated by deficiency of protein, androgen or estrogen. Whether calcium deficiency alone could affect the bone formation process or lead to demineralization in some other way would be difficult to demonstrate, at least in patients having the clear clinical criteria of osteoporosis.

Time permits only mention of some of the suggested bits of evidence which hint that calcium intake levels may have some importance in relation to the demineralization of osteoporosis. Of very modest value in this consideration are data obtained by dietary survey methods of the calcium intake habit of patients with senile and post-menopausal osteoporosis in comparison with normal individuals of the same age. Vinther-Paulsen (28) measured the habitual calcium intake of 33 inhabitants of an old folks home by weighing the diets these individuals selected over a period of 14 days. Among those taking less than 0.5 gm of calcium/day the incidence of osteoporosis was 74%; among those taking more than 0.5 gm, the incidence was only 14%. Smith (29, 30) in Detroit and Nordin (31) in Glasgow each with a much larger series have found, by dietary histories inquiring back many years, significantly lower levels of calcium intake in their osteoporotic patients than in non-osteoporotic control individuals of the same age. Nordin found the mean calcium intake in 72 cases of osteoporosis to be 13.4 ± 1.5 mg per kg per day, in 96 control subjects 17.9 ± 1.3 mg per kg per day. Smith found a mean daily calcium intake of 587 mg for 68 osteoporotic patients and 836 mg for non-osteoporotics. Smith's figures show a greater difference than do Nordin's given on a weight basis, probably because, as Smith has observed, osteoporotic patients weigh approximately 10% less than normal individuals. Significant differences were also found in both series for protein intake although less marked than for calcium intake, the differences roughly corre-

sponding to what would be expected on the basis of different levels of milk intake.

Another item bearing on the importance of calcium in these disorders, is the recent physicochemical work (32) at which Neuman's group has been so active. These studies have led to the formulation of concepts (33) which give calcium fully as important a place in calcification as the osteoid matrix; they stress the likelihood that there are important enzymatic processes in bone or plasma or both which regulate the shifts of mineral between a soluble phase in blood and the solid phase in bone in both bone formation and resorption; the rates of these processes being principally under hormonal control. Whether these processes, or one of them, are altered by

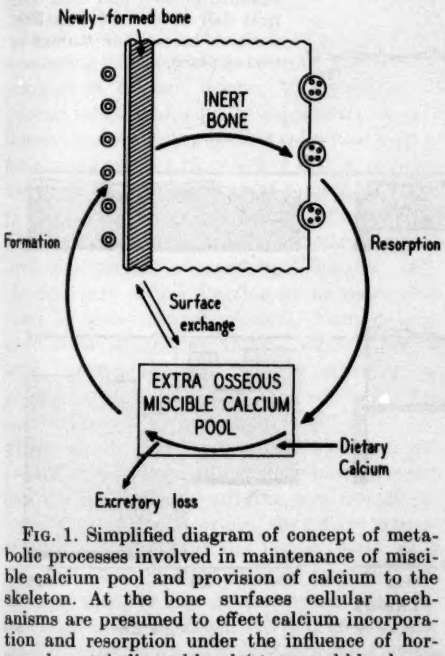


FIG. 1. Simplified diagram of concept of metabolic processes involved in maintenance of miscible calcium pool and provision of calcium to the skeleton. At the bone surfaces cellular mechanisms are presumed to effect calcium incorporation and resorption under the influence of hormonal, metabolic and local (stress and blood supply) forces. For the maintenance of serum calcium levels and of the miscible calcium pool the skeleton provides a ready storehouse of calcium through bone resorption, implying that resorption is a particularly active and easily variable process. In this scheme bone surface exchange is at present visualized as playing a smaller and less active role in calcium homeostasis. As the commodity transported by these various processes, calcium is abundant but not limitless in amount, a deficiency through excess excretory loss or inadequate provision via the intake being ultimately disclosed as demineralized bone.

TABLE 1. EFFECT OF ADDED CALCIUM ON CALCIUM STORAGE*

E.S., female, age 64, NYH-30326, osteoporosis

No. 7-day periods	Calcium intake, gm/day	Balance, average gm/day	
		Ca	N
3	1.480	−0.036	−0.53
2	4.210	+0.233	−0.17

* Shorr and Carter (39, p. 149.)

small changes in circulating calcium consequent to dietary or other alterations seems possible but is not yet known.

Radioisotopic studies are now beginning to shed both light and confusion on these matters. Using calcium-45, Bauer *et al.* (34) in Sweden, and Heaney (35) in our laboratory, devised a method of determining rates of calcium deposition in various diseases. Based on a number of important assumptions and on the finding that the miscible calcium pool is approximately equivalent in size to the extra-osseous or non-skeletal body calcium, rates of calcium leaving this pool, over several days' measurement, are assumed to represent rates of calcium deposition in forming bone. Correction for losses in urine and feces must, of course, be made. By this technique so-called 'bone formation rates' in Paget's disease and in clinical abnormalities of thyroid function show their expected aberrances from normal. In osteoporosis, however, 7 patients thus far studied have shown rates in the normal range. Similarly normal rates in osteoporosis have since been reported by Nordin (31, 36). This finding raises the possibility that increased bone resorption may contribute to the diminished bone mass in osteoporosis. Bearing in mind the idea that the skeleton is a great storehouse of calcium to be called on when calcium is needed for homeostatic control of pool and plasma levels, the concept (fig. 1) is suggested that in osteoporosis the calcium stores of the skeleton may have been gradually drained to make good losses from the pool through mandatory excretion and also in bone formation. The implication is raised that the degree to which the skeletal stores have had to be called upon may have been in proportion to relative insufficiency of calcium from dietary sources. This concept is not intended to exclude the importance of hormonal factors in the pathogenesis of this disease, but to imply that there is a dual set of influences on the development of osteoporosis, hormonal and nutritional.

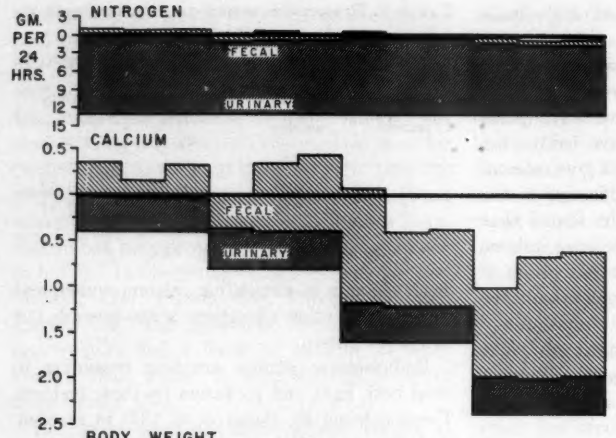


FIG. 2. Nitrogen and calcium balances and changes in body weight in *G.A.*, a 69-year-old woman with osteoporosis of the spine and active rheumatoid arthritis requiring administration of 15 mg prednisone orally throughout the study. In successive 18-day phases calcium intake was increased from 400 mg daily to 800 mg, to 1600 and to 2400 mg, plotted downward from the heavy base-line in this metabolic balance graph. When urinary and fecal outputs together rise above the base-line, negative balance or loss of the element is occurring; when the summed urinary and fecal outputs fall below the base-line, positive balance or storage is taking place.

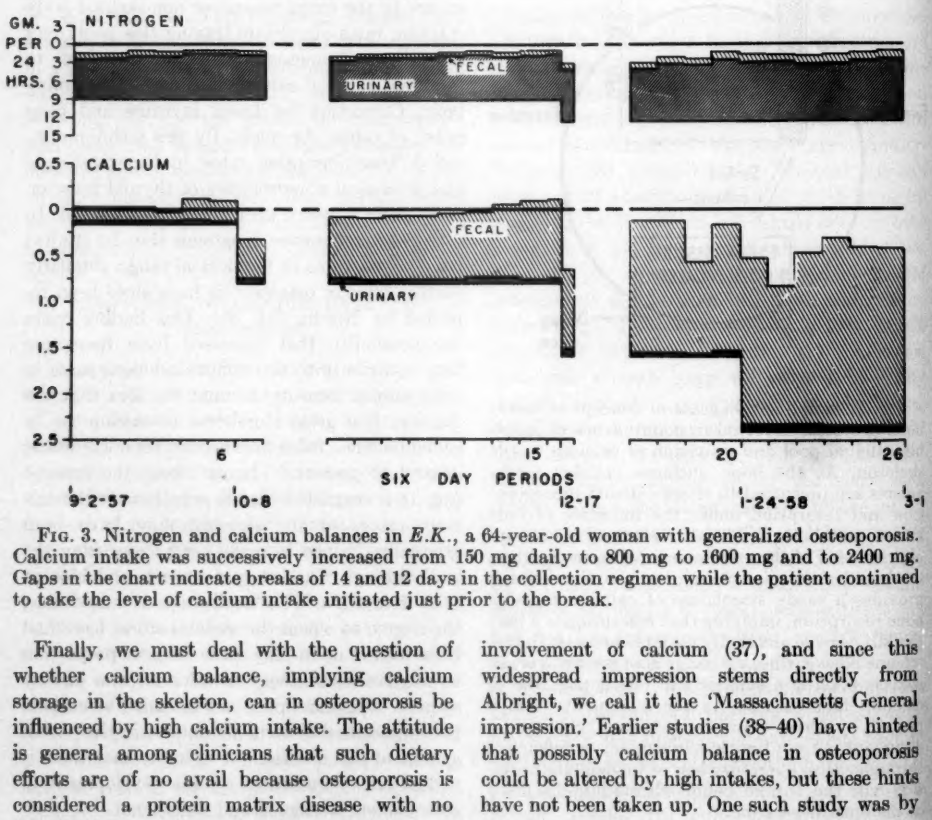


FIG. 3. Nitrogen and calcium balances in *E.K.*, a 64-year-old woman with generalized osteoporosis. Calcium intake was successively increased from 150 mg daily to 800 mg to 1600 mg and to 2400 mg. Gaps in the chart indicate breaks of 14 and 12 days in the collection regimen while the patient continued to take the level of calcium intake initiated just prior to the break.

Finally, we must deal with the question of whether calcium balance, implying calcium storage in the skeleton, can in osteoporosis be influenced by high calcium intake. The attitude is general among clinicians that such dietary efforts are of no avail because osteoporosis is considered a protein matrix disease with no involvement of calcium (37), and since this widespread impression stems directly from Albright, we call it the 'Massachusetts General impression.' Earlier studies (38–40) have hinted that possibly calcium balance in osteoporosis could be altered by high intakes, but these hints have not been taken up. One such study was by

TABLE 2. MEAN URINARY CALCIUM EXCRETION AT VARIOUS LEVELS OF CALCIUM INTAKE IN FIVE PATIENTS WITH OSTEOPOROSIS (MG/DAY)

Intake	G.S.	M.M.	K.C.	G.A.	E.K.	Average
150		106	88		31	
400		109	107	362		
800	174	122	116	402	45	172
1600	142	137	117	392	44	166
2400				400	63	

Shorr (39) who showed that a tremendous intake level of calcium would shift balances from negative to positive (table 1); note the fact that the patient was originally in negative balance at an intake level many would consider already quite high. Such studies may be criticized because of their comparative brevity.

In our own studies (41) we have recently been attempting to assess in osteoporotic patients the response of calcium balance to alterations in calcium intake and, equally importantly, to see, if response does occur, whether calcium storage is maintained over a long period of time or whether there may be a phenomenon of early 'saturation' of the exchangeable pool with calcium, then adjustment whereby the added dietary calcium load is diverted to excretion. Thus far, only the first part of this question seems answerable (that is, does response occur?). Five patients have been studied at various calcium intake levels between 150 and 2400 mg, generally for 18 days or more at a time, at each diet level. An example of such a study is shown in the graph (fig. 2) of the data on a 69-year-old patient who, in addition to generalized osteoporosis, had active rheumatoid arthritis, and throughout required an adrenal cortical steroid for control of joint symptoms; at the highest level, 2400 mg, the mean positive balance over the last 12 days was 650 mg. A more recent study in which we have lengthened the time at each diet level to from 36–72 days is shown on a graph (fig. 3) illustrating a six months' study of an uncomplicated osteoporotic 63-year-old female. The positive daily calcium balance during the last 24 days of the 36 on 2400 mg averaged 405 mg, a storage equivalent to the best obtainable with gonadal steroids. In table 2, for the five patients, the lack of significant change in urinary calcium at varying intake levels is shown. This observation is in agreement with previous findings that urinary calcium excretion does not reflect intake calcium with much sensitivity (42). Figure

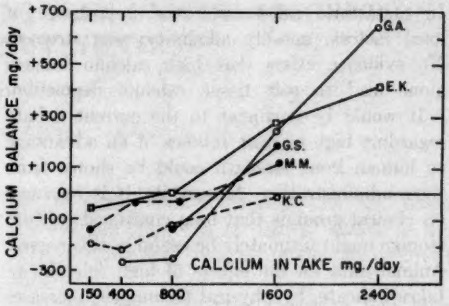


FIG. 4. Relationship between calcium intake and calcium balance in five patients with osteoporosis studied for 18 days or more at each intake level. Patient *G.A.*, in addition to osteoporosis, had active rheumatoid arthritis requiring 15 mg of prednisone daily throughout her study.

4, summarizing the balance data on these 5 patients, suggests three points: *1*) in osteoporosis calcium balance does vary with calcium intake; when calcium intake is raised patients will store substantial amounts of calcium at least for several weeks; *2*) considerable variation occurred from patient to patient in the intake level, from 800 to 1600 mg, at which the lines connecting the balance points cross the zero balance line (of some significance in respect to the question of calcium requirement); *3*) the mean intake level at which these lines cross the zero balance line is 1100 mg. It seems highly important now to determine whether positive calcium balances of considerable degree can be maintained over periods of months and months; the possibility that such may occur is suggested by the experience of Malm (4) with males on high intakes of much more modest levels. It may be of further interest in regard to this question, that calcium tolerance tests in some of our patients show greater retention of calcium as the intake level is raised (43), suggesting the possibility that high calcium intakes in these patients may activate or prime mechanisms either for storage of calcium or for inhibiting calcium resorption.

SUMMARY

No evidence is available that blood calcium levels rise significantly in response to ordinary dietary sources of calcium given on either an acute or chronic basis. Modest temporary rises are obtainable on the oral administration of the more soluble calcium salts.

The calcium content of soft tissues seems unaffected by wide variations in calcium intakes.

In metastatic calcification the importance of local factors, notably alkalinity, was stressed. No evidence exists that high calcium intakes alone lead to soft tissue calcium deposition.

It would be pertinent to the current debate regarding high calcium intakes, if an advantage to human bone strength could be shown from their administration. Meanwhile, it is reasoned on clinical grounds that long continued calcium storage might ultimately be useful in this regard. Animal data on the effects of high calcium intakes indicate, by physical techniques, increases in bone density; and human balance data in individuals without disease show a positive relationship between intake and balance through a wide range of intakes.

The possibility that this same positive relationship between calcium intake and balance is present in the demineralized condition of older people, osteoporosis, has been indicated in recent studies which may need to be lengthened. These studies suggest that the calcium requirement may vary widely from one individual to another and hint that in patients who develop osteoporosis the requirement may either be higher than for other individuals or that it may be higher than their customary intake over many years. Various bits of evidence (dietary survey, physico-chemical and isotopic) were cited which support the suggestion that calcium at abundant intake levels may play a significant role in the integrity of the skeleton of the aging human being.

REFERENCES

1. Shohl, A. T. *Mineral Metabolism*. New York: Reinhold, 1939, p. 134.
2. Schmidt, C. L. A. and D. M. Greenberg. *Physiol. Rev.* 15: 297, 1935.
3. Greenberg, D. M. and L. Gunther. *Arch. Internal Med.* 50: 855, 1932.
4. Malm, O. J. *Calcium Requirement and Adaptation in Adult Men*. Oslo: Oslo University Press, 1958.
5. *Nutr. Rev.* 14: 338, 1956.
6. Shelling, D. H., D. E. Asher and D. A. Jackson. *Bull. Johns Hopkins Hosp.* 53: 348, 1933.
7. Hansard, S. L. and M. P. Plumlee. *J. Nutrition* 54: 17, 1954.
8. Barr, D. P. *Physiol. Rev.* 12: 593, 1932.
9. Haas, G. M. In: *The Biochemistry and Physiology of Bone*, edited by G. H. Bourne. New York: Acad. Press, 1956, p. 775.
10. Bauer, W., A. Marble and G. A. Bennett. *Amer. J. med. Sci.* 182: 237, 1931.
11. Milk-alkali disease. *Nutr. Rev.* 13: 199, 1955.
12. Pasternak, B. *Doktor Zhivago*. New York: Pantheon, 1958, p. 63.
13. Hegsted, D. M. *Nutr. Rev.* 15: 257, 1957.
14. Bell, G. H., D. P. Cuthbertson and J. Orr. *J. Physiol.* 100: 299, 1941.
15. Sherman, H. C. and L. E. Booker. *J. Biol. Chem.* 93: 93, 1931.
16. Campbell, H. L., O. A. Bessey and H. C. Sherman. *J. Biol. Chem.* 110: 703, 1935.
17. Ellinger, G. M., J. Duckworth and A. C. Dalgarmo. *Brit. J. Nutrition* 6: 235, 1952.
18. Bauer, W., J. C. Aub and F. Albright. *J. Exper. Med.* 49: 145, 1929.
19. Schraer, H. and R. Schraer. *Fed. Proc.* 15: 571, 1956.
20. Ackroyd, W. R. *Lancet* 235: 153, 1938.
21. Thorangkul, D., F. A. Johnston, N. S. Kime and S. J. Clark. *J. Am. Dietet. A.* 35: 23, 1959.
22. Mitchell, H. H., T. S. Hamilton, F. R. Steggerda and H. W. Bean. *J. Biol. Chem.* 158: 625, 1945.
23. Leichsenring, J. M., L. M. Norris, S. A. Lamison, E. O. Wilson and M. B. Patton. *J. Nutrition* 45: 407, 1951.
24. Steggerda, F. R. and H. H. Mitchell. *J. Nutrition* 31: 407, 1946.
25. Ohlson, M. A., W. D. Brewer, L. Jackson, D. P. Swanson, P. H. Roberts, M. Mangel, R. M. Leverton, M. Chalonpka, M. R. Gram, M. S. Reynolds and R. Lutz. *Fed. Proc.* 11: 775, 1952.
26. Roberts, P. H., C. H. Kerr and M. A. Ohlson. *J. Am. Dietet. A.* 24: 292, 1948.
27. Blau, M., H. Spencer, J. Swernov, J. Greenberg and D. Laszlo. *J. Nutrition* 61: 507, 1957.
28. Vinther-Paulsen, N. *Geriatrics* 8: 76, 1953.
29. Smith, R. W., Jr., W. R. Eyler and R. C. Mellinger. *Ann. intern. Med.* In press.
30. Smith, R. W., Jr. In: Proceedings of the 1958 Lankenau Research Conference 'Bone as a Tissue'. New York: McGraw-Hill. In press.
31. Nordin, B. E. C. In: Proceedings of the 1958 Lankenau Research Conference 'Bone as a Tissue'. New York: McGraw-Hill. In press.
32. Neuman, W. F. and M. W. Neuman. *Am. J. Med.* 22: 123, 1957.
33. Howard, J. E. *J. Clin. Endocrinol.* 17: 1105, 1957.
34. Bauer, G. C. H., A. Carlsson and B. Lindquist. *Acta med. scandinav.* 158: 143, 1957.
35. Heaney, R. P. and G. D. Whedon. *J. Clin. Endocrinol.* 18: 1246, 1958.
36. Nordin, B. E. C. *Proc. Roy. Soc. Med.* 52: 351, 1959.
37. Bartter, F. C. *Am. J. Med.* 22: 797, 1957.
38. Adams, M., W. M. Boothby and A. M. Snell. *Am. J. Physiol.* 114: 383, 1935.
39. Shorr, E. and A. C. Carter. Tr. Second Conf. on Metabolic Interrelations. New York: Macy, 1950, p. 144.
40. Bogdanoff, M. D., N. W. Shock and M. P. Nichols. *J. Gerontol.* 8: 272, 1953.
41. Whedon, G. D. In: Proceedings of the IV Congress Intern. Assoc. of Gerontol. Fidenza, Italy: Tito Mattioli, 1957, Vol. II, p. 615.
42. Knapp, E. L. *J. Clin. Invest.* 26: 182, 1947.
43. Lutwak, L. and G. D. Whedon. *Clin. Res. Proc.* 7: 135, 1959.

EFFECTS OF HIGH CALCIUM INTAKES ON THE ABSORPTION OF OTHER NUTRIENTS

GEORGE K. DAVIS

Florida Agricultural Experiment Station, Gainesville, Florida

ONE OF THE MOST striking features of a serious review of the literature on 'Excess Calcium Intakes', is the paucity of firm data with respect to the effects of calcium upon absorption of nutrients other than calcium itself. There appears to have been abundant concern with the influence of increasing intakes of dietary calcium upon the utilization of calcium (1–14). Most investigations have shown that as the dietary calcium increases, percent absorption decreases, but total absorption and retention may increase. Animals appear to adjust more or less readily to the level of calcium intake (4, 5, 11, 15, 16). Such adjustment is reflected in a varying efficiency of absorption and to some degree, utilization. On the other hand, a consideration of the influence of high intakes of calcium upon the absorption of other ingredients leads one to the conclusion that there is a broad field which needs definitive study. H. A. Keener reported in 1953 (17) that "No information on the effect of excess calcium in the diet on the digestion of common feed ingredients was found in the literature." In 1959, the picture is not changed greatly, although there has been some additional work indicating that the effect of calcium additions may differ with different forage feeds (18). Our knowledge of zinc nutrition has resulted in a renewed interest in the problem of high calcium in the diet and the absorption of other nutrients. Work with manganese, iron, fat and iodine has also shown a relationship to this problem.

High intakes of calcium in the diet are not uncommon in animal feeding practice. In the human diet, high levels of calcium may occur, as it does in the polished-rice eating areas, where ground limestone is often used as a polishing agent. In laying hens, intakes above 1% are the rule. Perhaps laying birds should be considered as a class separate from other species and from non-laying birds, because of the very high calcium requirement for egg shell formation (12, 19–21). Nevertheless, it was shown more than 20 years ago that poultry suffer from high levels of calcium when associated with a manganese intake that is marginal (22, 23). When the calcium level was as high as 3.95% in the diet of laying hens, reduced production occurred (24).

While it has often been suggested that high levels of calcium, usually in the form of calcium carbonate, but also in the form of calcium phosphates, will reduce the digestibility of food and feed nutrients, the experimental data supporting such a statement have been very limited and the mechanism whereby calcium may have reduced the digestibility of other nutrients has been but poorly evaluated.

Keener, reporting to the Cornell Nutrition Conference in 1953, gave the results of two experiments with a limited number of animals in which he observed a consistent depression of protein digestion when the diets had approximately 1% of calcium carbonate added to them (17). A concurrent reduction in digestibility of energy was also noted. No attempt was made to define the exact method by which the calcium may have reduced the digestibility of these feed ingredients.

From a practical standpoint, adding extra limestone appeared inadvisable. On the other hand, these experiments do not give us much fundamental information upon which to evaluate the action of excess calcium on protein or some amino acids or upon the fat or carbohydrate portions of the diet. There is always the possibility that in the ruminant, increased levels of calcium may actually function through suppression of trace element availability in the rumen and thereby lower the efficiency of microorganisms in that organ. The end result would be a reduction in 'digestibility'.

Swenson and co-workers found that this overall effect of high calcium is apparent in the growth of beef cattle. Increasing the calcium to phosphorus ratio of the diet to 10:1 by addition of calcium carbonate caused a drop in weight gains from 302–225 pounds and, in a second experiment, from 548–490 pounds (25). No suggestion was made regarding the reason for this effect of added calcium.

There is some evidence that poultry given calcium, ad libitum, in the form of oyster shell and protein, ad libitum, in the form of a protein supplement will consume more protein with somewhat better performance than when the limestone or oyster shell is mixed with the protein supplement. Whether this improved performance with protein and calcium carbonate separated as opposed to that offered in the same feed mixture is due to differences in appetite or to differences in digestibility is not at present clear (26).

There may be an optimum protein intake for maximum utilization of calcium (27, 28). However, the available data do not justify the conclusion that calcium increases, in the diet, will improve the biological value of protein (28).

The only conclusion which one can draw with respect to the influence of high levels of calcium on protein utilization by animals and by humans is that with calcium intakes of approximately 0.5 to 0.8% of the diet, increasing the level of protein, favorably influences both the utilization of calcium and the utilization of the protein up to a maximum which appears to vary considerably with the different species.

There is somewhat more evidence that levels of calcium, exceeding 1%, in the diet may have an adverse effect upon growth in animals. In a series of papers from the Rowett Institute evidence was presented to show that addition of calcium carbonate to the diet of mice at a level of 1% sharply depressed the reproduction and growth of this species (29–31). These workers were not able to identify the mechanism whereby the calcium carbonate had this adverse effect. They presented evidence that the increased calcium may have prevented availability of some of the fat in the diet and also may have prevented iron absorption.

In work with rats some 10 years earlier than the reports from England, investigators in this country showed that increasing the calcium or the calcium and phosphorus levels of the diet caused a reduction in the deposition of fat in bones and the total body dry matter (32). A somewhat later publication by the same workers showed that reducing calcium from 1% to approximately 0.5% actually resulted in an increased absorption of food ingredients from the digestive tract. While by inference, the higher level of calcium was preventing feed utilization, the mechanism was not apparent. (32a)

The suggestion that high levels of calcium might be causing a depression of growth rate and/or a poor utilization of energy has attracted attention to the relationship between high calcium intakes and fat utilization. Perhaps the most outstanding work along this line has been that conducted by California workers in which they were able to show that increasing the level of calcium (0.6% added) and/or magnesium (0.09% added) resulted in a sharp depression in the digestibility of high melting point fats. This reduction in digestibility of fats by increasing the calcium level, amounting to over 50%, is of particular significance in this day of hydrogenated fats and high levels of fats in animal feeds (33). Although there was a very sharp reduction in digestibility of high melting point fats, the addition of extra calcium to the diets containing low melting point fats was without significant effect.

An earlier indication that increasing calcium and increasing calcium and phosphorus decreased the fat deposited in the skeleton of growing pigs came from Norfeldt (34), but it was not clear that this action was upon fat absorption.

It seems that our evidence for an effect of calcium on fat is restricted to an adverse effect by high levels of calcium upon high melting point fats. There is some evidence that increasing levels of fat in the diet will decrease the availability of calcium and this also is much more marked with high melting point fats (16, 35).

Richards (31) reported from England that an increasing level of calcium carbonate intensified a B vitamin deficiency in young rats which had been subjected to a deficiency diet. There is, however, no suggestion as to how this effect of calcium carbonate may have been exerted any more than it is clear why a high intake of calcium carbonate may depress egg production (24).

There is a tremendous amount of information upon the effect of high levels of calcium intake upon the utilization of calcium. In general, these results may be summed up as indicating that high levels of calcium increase the net retention of calcium by the animal but with a marked reduction in efficiency. There is also considerable evidence that there is an adaptation by different species to different levels of calcium intake. For this reason, calcium requirement figures may be more of a reflection of the dietary pattern than a reflection of absolute requirements. This has made interpretation of calcium data difficult

especially where the phosphorus intake has been retained at a constant level. There are strong indications that excess calcium will result in the formation of insoluble tricalcium phosphates and especially with marginal intakes of phosphorus may result in a phosphorus deficiency condition. Where higher levels of phosphorus accompany the higher levels of calcium, precipitation of a phosphorus deficiency is unlikely although the efficiency of phosphorus utilization may be very markedly reduced by the high levels of calcium intake. This brings into the whole pattern, the problem of calcium and phosphorus ratio; and with the increasing ratio there is a decreasing efficiency of utilization of phosphorus.

This is not the place to review the effect of calcium:phosphorus ratio on the development of rickets and upon the interrelationship of calcium and phosphorus with vitamin D_2 and D_3, but it does appear that high levels of calcium intake, especially those over 1% of the diet, will have a depressing effect upon phosphorus utilization (14, 15, 36–41).

Availability of magnesium is also markedly influenced by the presence of excess calcium in the diet. As with phosphorus, the adverse effect of high calcium upon magnesium is exerted only when the intake of magnesium is minimum. Again, it is not clear just how calcium and magnesium interact within the animal body, but at least part of the effect appears to be depressed magnesium absorption (5, 40, 42–44).

As with magnesium, the effect of excess calcium upon the trace elements iron, iodine, zinc, manganese and possibly copper have been observed experimentally and in every case where the intake of these elements is marginal or just adequate, with low intakes of calcium, increasing the calcium level of the diet has precipitated a deficiency.

An iron deficiency anemia has been produced, especially in rats and mice, where the diet has met the minimum requirement for iron but with high calcium levels. This iron deficiency anemia has been particularly apparent in young animals, but has also developed in breeding females under these dietary conditions. Other changes which have been associated with this high calcium intake have been identified as being caused by the reduced iron absorption with the resultant iron deficiency anemia. It would appear that increasing the intake of calcium will prevent iron absorption from the intestinal tract when the intake of iron is barely adequate to meet iron needs without added calcium. Under these circumstances of high calcium intake, it has been possible to meet the needs of the animal for iron by stepping up the iron intakes (24, 31, 40, 45–50).

In a similar fashion, calcium has been shown to have a goitrogenic effect and to sharply influence the need for iodine. There is a much less certain influence of calcium upon iodine absorption and although this has been proposed as a mechanism whereby calcium exerts its goitrogenic effect (51–53); more recent evidence (54) suggests that while calcium carbonate at a level of 2% in the diet exhibits a definite goitrogenic effect, this action of calcium carbonate is not through interference with iodine absorption. This is supported by histological observation of thyroids of rats on similar diets (55). While calcium carbonate, at high levels, definitely serves to increase the need for iodine such a need is readily met even with high calcium intakes by increasing the iodine in the diet.

The discovery that manganese was necessary to prevent perosis in poultry and the subsequent determination that manganese was necessary for normal bone structure and formation in swine and rabbits has focused attention upon the relationship between manganese and the level of calcium and phosphorus in the diet. It was early illustrated in these three species that increasing calcium and/or phosphorus in the diet intensified the manganese deficiency and the development of perosis. The known relationship between calcium and manganese in plant nutrition suggested that the increased levels of calcium were suppressing the solubility of manganese and rendering the manganese unavailable for absorption in the intestinal tract. While this may be the mechanism of action, there is no clear-cut evidence as to just how or where the excess calcium suppresses manganese solubility and/or availability to the animal; and as is the case with iron and iodine, increasing the level of manganese in the diet will overcome the adverse effect of high levels of calcium and calcium and phosphorus (21, 22, 41, 56–59).

Perhaps no other discovery has focused attention upon the effect of high levels of calcium in the diet than the discovery that swine develop a severe parakeratosis when the calcium in the diet is increased well above requirement levels without concurrently increasing the zinc level of the diet. With adequate levels of calcium, but

not excessive levels, swine do very well with zinc intakes of between 30 and 40 ppm. When the calcium level is increased to over 1%, a sharp depression of growth occurs and in a high proportion of the pigs on this diet parakeratosis develops. Under these circumstances either reducing the calcium intake or increasing the zinc intake will prevent the development of the abnormal symptoms. Again it is not clear how the calcium adversely effects the absorption or utilization of zinc. While there is some evidence that the absorption is reduced by a change in pH in the areas of the intestine where absorption is most active (50), this is far from clear and the mechanism of calcium suppression of zinc utilization remains a problem for study (60–63).

Increasing calcium in the diet above 1% may have a sharp depressing effect upon the utilization of other nutrients in the diet including protein, fats and the vitamins; the macromineral elements, phosphorus and magnesium; and the trace mineral elements especially iron, iodine, zinc and manganese. In those instances where the intake of these nutrients is limited, increasing the calcium of the diet may have a markedly adverse effect and may produce a clinical deficiency of the other nutrients. There is as yet no clear picture as to just how the excess calcium may exert its influence. It is possible that the high level of calcium, especially in the form of calcium carbonate may cause an increase in the pH of the intestinal tract thereby changing solubilities. There may be a formation of insoluble compounds and such a mechanism appears to be favored with respect to the action of calcium upon phosphorus, on fat and on iron. Perhaps if this review has done nothing else, it has suggested that much more research must be carried out before the recognized adverse action of high levels of calcium in the diet can be explained in terms of specific actions within the intestinal tract.

REFERENCES

1. Causeret, J. and D. Hugot. *Ann. Inst. nat. recherche agron: Série C.* 7: 69, 1958.
2. Palmer, R. F., R. C. Thompson and H. A. Kornberg. *Science* 127: 1505, 1958.
3. Fisher, K. H. and M. L. Dodds. *J. Am. Dietet. A.* 34: 392, 1958.
4. Gershoff, S. N., M. A. Legg and D. M. Hegsted. *J. Nutrition* 64: 303, 1958.
5. Hollinger, H. Z. and C. J. Pattee. *Canad. M. A. J.* 75: 941, 1956.
6. Howard, J. E. *J. Clin. Endocrinol.* 17: 1105, 1957.
7. Wasserman, R. H., C. L. Comar and D. B. Papadopoulou. *Science* 126: 1180, 1957.
8. Blau, M., H. Spencer, J. Swernov, J. Greenberg and D. Laszlo. *J. Nutrition* 61: 507, 1957.
9. Hansard, S. L., C. L. Comar and G. K. Davis. *Am. J. Physiol.* 177: 383, 1954.
10. Hansard, S. L. and M. P. Plumlee. *J. Nutrition* 54: 17, 1954.
11. Migicovsky, B. B. and J. W. S. Jamieson. *Canad. J. Biochem. & Physiol.* 33: 202, 1955.
12. Wilcox, R. A., C. W. Carlson, W. Kohlmeyer and G. F. Gastler. *Poultry Sci.* 32: 1030, 1953.
13. Fournier, P. *C. R. Acad. Sci.* (*Paris*) 232: 1593, 1951.
14. Shohl, Alfred T. and S. Farber. *J. Nutrition* 21: 147, 1941.
15. Mitchell, H. H. *Mod. Med.* 23: 85, 1955.
16. Nicolaysen, R., N. Eeg-Larsen and O. J. Malm. *Physiol. Rev.* 33: 424, 1953.
17. Keener, H. A. Cornell Nutrition Conference, 1953.
18. Byers, J. H. *J. Dairy Sci.* 42: 935, 1959.
19. O'Rourke, W. F., P. H. Phillips and W. W. Cravens. *Poultry Sci.* 34: 47, 1955.
20. Patrick, H. and G. K. Schweitzer. *Poultry Sci.* 32: 920, 1953.
21. *Nutrient Requirements of Poultry. Nat. Research Council* (*U. S.*), Pub. No. 301, 1954.
22. Wilgus, H. S., L. C. Norris and G. F. Heuser. *Science* 84: 252, 1936.
23. Wilgus, H. S., L. C. Norris and G. F. Heuser. *J. Nutrition* 14: 155, 1937.
24. Gutowska, M. and R. T. Parkhurst. *Poultry Sci.* 21: 321, 1942.
25. Swenson, M. J., G. K. L. Underbjerg and D. D. Goetsch. *Am. J. Vet. Res.* 17: 207, 1956.
26. Johnson, E. A. *Poultry Sci.* 33: 1063, 1954.
27. Greenberg, R. M., M. G. Barry and J. Mayer. *Fed. Proc.* 11: 444, 1952.
28. Henry, K. M., S. K. Kon and S. Y. Thompson. *Biochem. J.* 34: 998, 1940.
29. Richards, Marion B. *Brit. J. Nutrition* 3: 109, 1949.
30. Howie, J. W. and G. Porter. *Brit. J. Nutrition* 4: 175, 1950.
31. Richards, Marion B. and W. A. Greig. *Brit. J. Nutrition* 6: 265, 1952.
32. Haldi, J., G. Bachmann, W. Wynn and C. Ensor. *J. Nutrition* 18: 399, 1939.

32a. Haldi, J., W. Wynn and C. Ensor. *J. Nutrition* 20: 145, 1940.

33. Cheng, A. L. S., M. G. Morehouse and H. J. Deuel, Jr. *J. Nutrition* 37: 237, 1949.
34. Norfeldt, Sam. *J. Nutrition* 31: 565, 1946.
35. Kane, G. G., F. E. Lovelace and C. M. McCay. *J. Gerontol.* 4: 185, 1949.
36. Cramer, J. W. and H. Steenbock. *Arch. Biochem.* 63: 9, 1956.
37. Arrington, L. R. and G. K. Davis. *J. Nutrition* 55: 185, 1955.
38. Lewis, J. K., W. H. Burkitt and F. S. Willson. *J. Animal Sci.* 10: 1053, 1951.
39. Common, R. H. *J. Agr. Sci.* 30: 113, 1940.

40. De, H. N. and K. P. Basu. *Indian J. M. Res.* 37: 213, 1949.
41. Maynard, L. A. and J. K. Loosli. *Animal Nutrition.* New York: McGraw-Hill, 1956, p. 115.
42. Maynard, L. A., D. Boggs, G. Fisk and D. Sequin. *J. Nutrition* 64: 85, 1958.
43. Hogan, A. G., W. B. House and W. O. Regan. Missouri Agricultural Exper. Sta. Res. Bull. No. 567, 1954.
44. Hegsted, D. M., J. J. Vitale and Helen McGrath. *J. Nutrition* 58: 175, 1956.
45. Chapman, D. G. and J. A. Campbell. *Brit. J. Nutrition* 11: 117, 1957.
46. Chapman, D. G. and J. A. Campbell. *Brit. J. Nutrition* 11: 127, 1957.
47. Chapman, D. G. and J. A. Campbell. *Brit. J. Nutrition* 11: 133, 1957.
48. Greig, W. A. *Brit. J. Nutrition* 6: 280, 1952.
49. Kletzien, S. W. *J. Nutrition* 19: 187, 1940.
50. Anderson, H. D., K. B. McDonough and C. A. Elvehjem. *J. Lab. & Clin. Med.* 25: 464, 1940.
51. Keith, W. D. *Canad. M. A. J.* 14: 284, 1924.
52. Cameron, A. T. *Recent Advances in Endocrinology.* London: Churchill, 1947.
53. Buydens, M. R. *Bull. Acad. roy. Méd Belg.* 18: 186, 1953.
45. Taylor, S. *J. Clin. Endocrinol.* 14: 1412, 1954.
55. Sampson, M. M. and Ann Putzki. *Fed. Proc.* 11: 455, 1952.
56. Wachtel, L. W., C. A. Elvehjem and E. B. Hart. *Am. J. Physiol.* 140: 72, 1943.
57. Keith, T. B., R. C. Miller, W. T. S. Thorp and M. A. McCarty. *J. Animal Sci.* 1: 120, 1942.
58. Kemmerer, A. R. and W. R. Todd. *J. Biol. Chem.* 94: 317, 1931.
59. Maynard, L. A. and J. K. Loosli. *Animal Nutrition.* New York: McGraw-Hill, 1956, p. 152.
60. Klussendorf, R. C. *Vet. Med.* 51: 301, 1956.
61. Leucke, R. W., J. A. Hoefer, W. S. Brammel and D. A. Schmidt. *J. Animal Sci.* 16: 3, 1957.
62. Tucker, H. F. and W. D. Salmon. *Proc. Soc. Exper. Biol. & Med.* 88: 613, 1955.
63. Smith, W. H., M. P. Plumlee and W. M. Beeson. *Science* 128: 1280.

DISCUSSION

D. Mark Hegsted

Harvard School of Public Health, Department of Nutrition, Boston, Massachusetts

Perhaps the most remarkable thing about this symposium is the title and that it is a part of the annual meetings of the American Institute of Nutrition. It is probable that this is the first time that any nutrition society considered the possible detrimental effects of a high calcium intake, a topic worthy of discussion. The fact that the symposium was organized indicates that someone thought the subject to be of some importance and the papers we have heard today justify the supposition. Still many of you must feel, as I do, somewhat surprised, considering the efforts nutritionists in general have made to increase the consumption of calcium containing foods.

This is not the place to review the data upon calcium requirements. However, we may note that it is repeatedly claimed that 'calcium deficiency' is the principal deficiency in the population of the United States. This conclusion is reached by a comparison of dietary intakes with the recommended Dietary Allowance for calcium. Of course, in many parts of the world the percentage of the population that is 'calcium deficient' by this criteria is essentially 100 and one might suppose that they would be facing a terrible nutritional problem. Such is not the case. I feel that we have shown (1–3) by experimentation and logic that the current estimates of calcium need are the result of a faulty interpretation of balance data. The facts are that we do not know what the calcium requirement is and balance data are not likely to tell us what it is. Neither do we have at the moment, as far as I know, methods to determine the requirement with accuracy. Certainly the best evidence that it may be considerably lower than current estimates is the excellent performance which is seen throughout the world in peoples eating low calcium diets provided other problems, nutritional or otherwise, do not seriously complicate the picture.

In looking at today's symposium, one would probably have to conclude that the 'low calcium' and 'high calcium' groups reach a draw. If no one can point to serious detrimental effects in human beings as a result of the usual low calcium diet, neither is there convincing evidence that the habitual consumption of high calcium diets is detrimental. When one turns to specific disease states the situation is somewhat different. Here it seems to me that those who look with askance at

high calcium diets have the better of the argument at the moment. I take it that at least in three well-known situations—in 'stone formers,' in the milk alkali syndrome and in the hypercalcemias—a low calcium diet is rather clearly of therapeutic value. This in itself does not prove that high calcium consumption is a primary etiologic factor.

Perhaps one thing that should be clear to us all is that people are different and not only are they different but the way they respond to various nutrients depends upon many things including other factors in the diet. We are presumably looking for means to identify susceptible individuals and the conditions which influence this susceptibility. It seems to me that for the above listed diseases, calcium must be one of the important factors.

On the other hand, the same logic in reverse applies to osteoporosis. With loss of bone mineral, it seems only logical to supply high levels of calcium to try and prevent this loss. Again, the evidence that low calcium intakes are an important etiological factor, or that high calcium intakes are important therapeutically, leaves much to be desired. It would seem that if low calcium diets were important in the causation of osteoporosis, strong evidence could be obtained from those numerous populations which habitually consume low levels of calcium. I do not know how adequate the epidemiologic evidence is, but since we do not hear that osteoporosis is a more pressing problem in such areas, I assume that this argues strongly against low calcium consumption as a causative factor.

As usual, and as Dr. Davis has demonstrated, our knowledge in animals is much superior to our knowledge in man. There is little reason to doubt that the interrelations he has described, or similar ones, may pertain in human nutrition. It is perhaps to be expected that more extensive studies of the trace minerals in man, which are now only beginning, will reveal those of importance. Studies with animals in our laboratories have revealed a situation which is pertinent to today's discussion. Animals fed a pyridoxine deficient diet develop a marked oxaluria and these animals develop oxalate kidney stones (4, 5). In such animals it can be easily demonstrated that high calcium diets are detrimental. With more dietary calcium they develop larger stones and develop them more rapidly. Since there is evidence that the administration of pyridoxine to human subjects consuming a presumed adequate diet may lower the level of urinary oxalate excretion (6), these findings may have some relevance to human nutrition.

The conclusions which I draw from today's symposium are that there are possible detrimental effects from high calcium intakes. The evidence is not strong that a substantial proportion of the population of the United States is influenced adversely by the intakes they usually consume but better methods and more information might modify this conclusion. At the same time, there is no evidence that benefits will be produced by increasing the calcium intake still further, an objective of many nutritional education campaigns. There may well be as many people who consume levels of calcium considerably above the Recommended Allowances as there are who do not consume as much as recommended.

It would appear that the only logical course under these conditions is one of moderation. The great emphasis that nutritionists have placed upon calcium in the past is not justified by current information and has undoubtedly been one of the factors which has tended to push the calcium intake to high levels. It is time to take the pressure off of calcium as our major nutritional problem and there should be appropriate changes in our recommendations.

REFERENCES

1. Hegsted, D. M., I. Moscosco and C. Collazos. *J. Nutrition* 46: 181, 1952.
2. Hegsted, D. M. *Symposium on Methods for Evaluation of Nutritional Adequacy and Status.* Department of the Army, Office of the Quartermaster General. Dec. 1954, p. 103.
3. Gershoff, S. N., M. A. Legg and D. M. Hegsted. *J. Nutrition* 64: 303, 1958.
4. Gershoff, S. N., F. F. Faragalla, D. A. Nelson and S. B. Andrus. *Am. J. Med.* 27: 72, 1959.
5. Gershoff, S. N. and F. F. Faragalla. *Fed. Proc.* 18: 526, 1959.
6. Gershoff, S. N., A. L. Mayer and L. I. Kulczycki. *Am. J. Clin. Nutrition* 7: 76, 1959.

AMERICAN INSTITUTE OF NUTRITION

Symposium on Protein Requirement and Its Assessment in Man[1]

Chairman: PAUL GYÖRGY

INTRODUCTORY REMARKS

P. GYÖRGY

THE FOLLOWING SYMPOSIUM represents a deviation from the usual technique and should be considered in the same as a trial and rehearsal for symposia planned as the backbone of the program for the Fifth International Congress on Nutrition to be held in Washington, D. C., September 1960.

In the past symposia or panel discussion have usually consisted of elaborate presentations, from 20 to 40 minutes by the panelists, often followed by spontaneous, unorganized discussion. For the latter usually the time available became rather restricted. For the present symposium, emphasis is put on organized discussion and not on the single presentation. This should be achieved by pre-printing and distribution of the contributions, as elaborate and detailed as the panelists select to have. The Moderator has received in advance a set of questions from each panelist, pertaining to their own topic and to that of the other panelists. The Symposium itself will then consist of a short, precise summary by each panelist of their papers followed by discussion with the Moderator acting as the leader. Written questions from the floor will be solicited and used by the Moderator at his discretion. It is hoped that this technique will permit lively and detailed discussions, grouped in an organized fashion around special problems taken in relation to the major topic of the Symposium. This should avoid repetition and disorganized discussion and should also permit formulation of conclusions in which all, or at least the majority of panelists, may concur.

The selection of the topic of the present Symposium was almost self-evident. Protein nutrition is in the forefront of interest not only in nutritional research but also as world-wide problem of public health and to some extent also in clinical medicine. Problems of protein requirement, optimal protein-nutrition, the existence and role of protein reserve—to name only a few—are intensively debated and are still open questions. It is hoped that the present Symposium will at least act as a powerful ferment for further research in protein nutrition. And last but not least, the technique here applied should make the Symposium more lively, interesting as well as stimulating.

NITROGEN CONTENT OF THE BODY AND ITS RELATION TO RETENTION AND LOSS OF NITROGEN[2]

WILLIAM M. WALLACE

Department of Pediatrics, Western Reserve University School of Medicine, and Babies and Children's Hospital, Cleveland, Ohio

IN 1914 BENJAMIN (1), by an ingenious technique avoiding the immobilizing effect of the traditional metabolism frame, measured the nitrogen balances of a well-nourished and an undernourished infant for a continuous period of 70 days. During this interval human milk, human milk fortified with skimmed cow's milk, and whole cow's milk were used to supply in sequence feedings of variable protein concentration. He then calculated the nitrogen retentions in terms of 100 gm of weight gain made by the

[1] Atlantic City, N. J., April 15, 1959.

[2] Supported in part by Grant #A-2384 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

infants. For the normal infant the found retention on breast milk was 3.7/100 gm, on fortified breast milk 4.5/100 gm, and on whole cow's milk 5.3/100 gm. The undernourished infant's gains of nitrogen per 100 grams of weight gain on the 3 feedings were: 3.9 gm, 4.9 gm and 7.1 gm, respectively. His data indicated that the rise in retention with increase in protein intake was well sustained.

Benjamin subjected his data to further analysis by factoring the retentions in terms of body composition. He considered that the body contained 3.4% nitrogen and therefore, using the factor of 29.5, calculated the theoretical gain of weight on breast milk to be 109 gm, on fortified milk 133 gm and on cow's milk 156 gm. He concluded that breast milk is the ideal milk for infants because it most nearly produces 'correlative' weight gain. Forty-five years later the consensus remains the same although the supporting evidence seems no longer drawn from the techniques of the natural sciences. The author spends many pages considering the inexplicable finding of a gain of protein not represented by an appropriate gain of body weight. He considers at length the possibility that changes in body water account for the apparent storage of nitrogen, but ultimately discards this possibility and fails to explain the enigma.

Benjamin's value for the nitrogen content of the infant's body was that of the fat-free adult. Had he used the more nearly correct concentration of 2% for the infant of this age, his factor would have been 50 and correlative growth would not have occurred at any level of protein intake.

In the ensuing years the technique of nitrogen balance has been used intensively to assess the elusive problem of the optimal protein intake for growth and for repair of malnutrition. Almost without exception the findings have been the same as those of Benjamin's, i.e. the higher the protein intake, the greater is the apparent retention of nitrogen per unit of weight gain. Improvement in analytical techniques, in collection methods and in the application of modern statistical methods to the analysis of the data have lowered the apparent discrepancy but not the essential interpretation. The conclusion from such data has been widely accepted that higher protein intakes lead to an increased protein content of the whole body. The general inference has been that this is beneficial. Wide acceptance and use of the term 'protein depletion' is tacit evidence in this belief.

Truly significant variation in the protein content of the whole body is difficult to reconcile with knowledge of the actual nitrogen content of the whole bodies of both man and animals. When calculated on a fat-containing basis, apparently significant differences do occur but on a fat-free basis the nitrogen content is remarkably constant in all mammalian species. As will be indicated below, changes in body fat, a relatively small fraction of the body weight, cannot impressively alter the correlative interpretation of nitrogen retention or loss to weight change. Even starvation or surfeit feeding do not appear to alter the nitrogen content of the body to a degree that is significant for the interpretation of balance data. Dehydration and water excess can, when extreme, alter the whole body protein content to a significant extent. Such deficits or surpluses are borne, to the greatest extent, by the nonparenchymal fractions of the body mass. The water to protein ratio of the cellular tissues appears to be susceptible to much less variation.

Direct analytical data on the whole bodies of human infants and adults are too few to subject to satisfactory analysis of variability with regard to chemical elements. The obvious technical, legal and medical problems of obtaining such data on normal individuals are evident. The available modern data have been collected and compared elsewhere (2). These indicate an average value of 2% nitrogen for the whole body of the infant and 3% for the average adult.

Animal data on the nitrogen content of the body under varying conditions are abundant and indicate the relative constancy of the nitrogen content of the fat-free body. It is difficult to escape the conclusion that the significance of the constancy in animals also applies to the human species.

Table 1 is taken from the data of Wallace, Weil and Taylor (2) obtained by direct analysis of the whole bodies of albino rats allowed to grow for equal lengths of time on food with widely variable protein and ash contents. It is evident that the protein and ash contents are relatively constant and that what variation does occur, does not easily relate to the level of intake.

Table 2 is taken from the data of Widdowson and McCance (3). The experiments of these authors were designed to test the effects on body composition of equal losses of weight due to undernutrition and total fasting. Neither type of nutritional deprivation appears to lead to signifi-

cant change in the protein content on a fat-free basis.

Table 3 is taken from the direct carcass analysis values of Baur and Filer (personal communication) for groups of pigs fed variable protein rations during early growth and subjected to subsequent starvation with and without a water intake. While the control data for the high and low protein fed animals indicate different nitrogen concentrations, this difference is small, 1%, and, as will be discussed below, probably unmeasurable by the balance technique. Fasting alone does not appear to alter the fat-free body protein concentration. What differences are produced are related more to the state of hydration of the animal.

The data cited above indicate a high degree of constancy for the essential body composition of the animal under conditions of deprivation and variable protein intake. Absolute lean body weight and absolute body fat content may be changed but the concentration of nitrogen in the lean body appears to be quite fixed. These observations indicate that nitrogen balance data that do not bear reasonable extrapolation to predict body weight and body composition require scrutiny with regard to the validity of any conclusions drawn from them.

It is of interest to examine the factors used to convert nitrogen retention data to expected body composition and the possibilities of error in such a validating process. Five additional specific items require examination, i.e. the factor used to convert nitrogen to protein, the ash content of the body, the role of the water content of the body and the body fat content, and the possibility of cumulative error in the balance technique.

The collective body proteins as found in the proportion existing in the whole body contain 16% nitrogen. It is from this percentage that the familiar conversion factor of 6.25 is derived. Certain individual proteins may vary considerably from this percentage value but the body content of these is too small to affect the over-all value. The validity of this factor for the albino rat under conditions of varied protein intake has been examined elsewhere (2). The extensive analytical data of Baur and Filer (personal communication) for the pig also indicate the essential reliability of this factor for conversion of retentions to gain or loss of protein to the body.

The ash content of the body essentially measures the quantity of fat-free, protein-free bone mineral. This varies from species to species and from age to age within the species. In man the fat-free whole body ash content increases from about 3% at birth to as high as 7% in the adult male. The total relative quantity of bone mineral in the body is, however, too small to affect the interpretation of nitrogen balance data at any level of technical accuracy.

To explain the increasing nitrogen retention with increasing protein intake shown in the data of Benjamin and many subsequent investigators, it is reasonable to postulate a decreasing fat content of the body if the fat-free body is to retain a constant nitrogen concentration. No observa-

TABLE 1. EFFECT OF PROTEIN AND ASH INTAKE UPON BODY WEIGHT, PROTEIN AND ASH CONTENT OF THE FAT-FREE BODY OF THE ALBINO RAT

	Intake		Fat-free Body		
No. of Animals	Protein	Ash	Protein	Ash	Total Body Weight
	%	%	%	%	
8	23.4	6.02	20.23	3.50	151.8
12	23.4	3.08	19.40	3.10	149.7
12	12.0	6.02	18.55	4.09	97.7
8	12.0	3.08	20.30	4.09	102.9

From data of Wallace, Weil and Taylor (2). For statistical evaluation of above values see original data.

TABLE 2. EFFECT OF UNDERNUTRITION AND STARVATION UPON THE PROTEIN CONTENT OF THE FAT-FREE BODY OF THE RAT*

No. of Animals	Age and Sex	Controls	Undernourished	Starved
6	Adult male	27.2	25.0	26.6
6	Adult female	24.4	24.2	25.0
6	Young male	21.1	21.6	24.0
6	Young female	22.2	22.6	24.4

From data of Widdowson and McCance (3). No measure of variation given in original data.
* Values in percentage.

TABLE 3. EFFECT OF VARIABLE PROTEIN INTAKE AND SUBSEQUENT STARVATION UPON THE MEAN FAT-FREE BODY PROTEIN CONCENTRATION OF THE YOUNG PIG*

Protein of Intake	Starvation Controls	Starvation Water ad lib.	Starvation Water Deprived
13.75	18.1	15.9	21.3
	(12)	(3)	(5)
25.00	19.2	16.7	21.6
	(8)	(4)	(5)

From data of Baur and Filer (personal communication).
Numbers in parentheses indicate number of animals in the group.
* All values in percentage.

tions exist to indicate that such does occur to a degree sufficient to explain the retention data. In a very general way animals fed high protein intakes appear to have less body fat than do animals on low protein feedings (2). However, the degree of leanness is never sufficient to explain the extremely high retentions so often found during growth on high protein intakes. A simple calculation will illustrate the small effect of large changes in body fat content on the actual retention of nitrogen. From the age of 1 month to 3 months the human infant gains 1500 gm, a 35% increase in weight. If his body fat remains constant at 15% and the nitrogen concentration increases from 2.0% to 2.5% *vide infra*, he must gain 50 gm of nitrogen. His mean nitrogen retention for the interval will be 1.01 gm/kg. If he decreases his body fat content to 10% during the period, he will gain 57 gm of nitrogen and increase his per kilogram retention to 1.15 gm. If 4–8 day balance periods are utilized, it is evident that this small difference could not be measured. Like the ash content of the body, the body fat is too small a fraction of the body weight to affect nitrogen balance measurably.

Changes in body water have been invoked to explain the retention of nitrogen in excess of correlative body growth. Changes of normal body hydration in excess of 4–5% occur only in very abnormal clinical conditons and belong more in the realm of body fluid physiology than in that of nutritional evaluation of protein adequacy. However, the maturation of the body water to protein ratio that occurs during growth does have some relevance to the argument under consideration. At birth the whole body of the human infant contains about 2% nitrogen, while at so-called 'chemical maturity' it contains 3%. According to the data of Moulton (4), this maturation is completed in about $\frac{1}{20}$ of the lifespan, or at 3–4 years of age for the child. Ninety per cent of the maturation is complete by the age of 1 year. Figure 1 illustrates the calculated theoretical nitrogen retention in mg/kg of body weight per

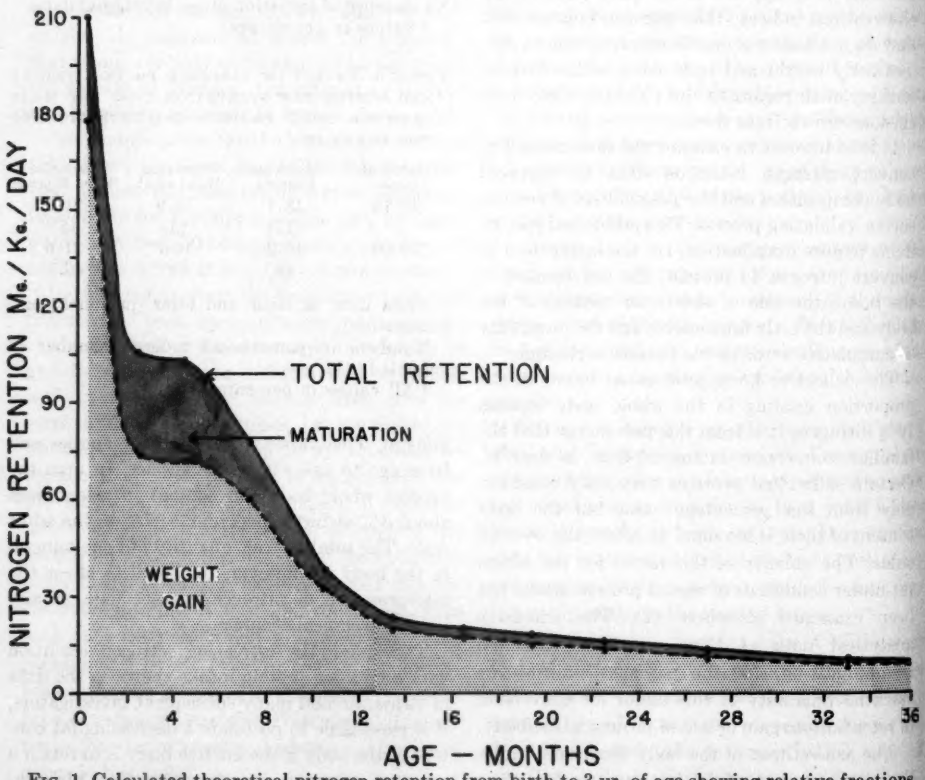


FIG. 1. Calculated theoretical nitrogen retention from birth to 3 yr. of age showing relative fractions of retention assignable to growth and to maturation of body composition. See text for sources of data and method of calculation.

day at various ages during the period of growth and body composition maturation. The body weights used to make the calculations are the 50 percentile figures for boys taken from the Harvard School of Public Health curves. It has been assumed that the fat content remains constant at 16% and that the nitrogen content increases from 2 to 3% during the interval. The percentage of nitrogen at different ages is taken from Moulton's curve (4). The lower curve indicates the retention required for increment in body weight alone, while the top curve indicates the total theoretical retention and includes the nitrogen retained to 'mature' the body composition. The area between the curves indicates the quantity required for this latter process. At the age of 4 months 33% of the retained nitrogen is being stored without equivalent gain of body weight. After this age this fraction rapidly decreases so that by the age of 4–5 years any gain of nitrogen should be represented by a reasonably predictable gain in body weight. Whether or not high protein intakes can speed up the process of body maturation in the growing child is not known.

The final item, that of positive cumulative error in the balance technique and calculation, is more difficult of precise analysis yet probably the most pertinent of the factors requiring consideration. An obvious and often considered error in the balance technique are skin losses of the substance in question. Available measurements of the nitrogen lost in this fashion range from 0.1 to 0.4 gram per m^2 per day. This rather great variation is probably a result of the various investigators' methods for removing the substance for analysis. Even when the largest of these estimates is applied to many nitrogen balance data correlative retention cannot be obtained. A more cogent source of error is inherent in the process of subtraction of outgo from input to obtain the final balance. Even with the most refined and meticulous technique a finite quantity of the measured intake is lost in the process of feeding and, similarly, a finite portion of the excreta is not recovered. When output is subtracted from intake, the two losses are additive, not self-cancelling as is generally supposed. The problem may be stated in another way: the numerical value obtained for intake is always larger than actuality while the numerical value for output is always smaller. Subtraction of the two numerical values compounds the error.

It should be emphasized that this type of error is distinct from the random type of error inherent in the chemical method used to analyze the two components of the balance. Because this error is unknown, it cannot be subjected to statistical analysis. At first glance this error seems negligible, particularly when viewed against the magnitude of the total exchanges involved. However, when it is considered that two very large numerical values are subtracted to obtain what is usually a very small yet highly significant balance value this compounded error becomes highly significant. Thus, when a high protein containing milk is fed an infant who is having the usual retention of nitrogen for growth, a 2% loss of intake and a similar loss of excreta will result in a 30–40% increase in the determined balance. Failure to include skin losses may increase the error by at least a further 20%. When carrying out balances, the quantity of a substance under study may be widely variable while the actual volumes or weights of the intake and output are more nearly constant. The comparison of the nitrogen exchanges when breast milk and cow's milk are fed is a pertinent example. Equal volumes contain very different nitrogen contents. The higher the concentration of the substance in the food fed, the greater will be the losses and the greater the error in the balance. There can be little doubt that the high retentions of nitrogen per unit of weight gain found on high protein containing milks and foods find at least a partial explanation in this source of error. As such, they make conclusions drawn from such data of dubious validity. The conclusion may be correct but the reason for the conclusion is erroneous. Careful estimations of body weight and length, of body fat by the skin caliper technique or by indirection from body water measurement or by specific gravity would seem to be more logical ways to assess body composition during protein feeding trials.

SUMMARY

The difficulty encountered in reconciling retentions of nitrogen with the actual composition of the body has been shown. Data have been presented to show that the nitrogen concentration in the fat-free body is always relatively constant despite extreme variation in previous dietary experience and despite marked changes in body weight. The factors used to convert nitrogen retentions to expected body composition and size and the effect of possible changes in body fat and

body water upon the interpretation of nitrogen retention data have been presented. The magnitude of the additive errors inherent in the method of calculating balances has been indicated and their very large role in causing lack of correlation between retentions and true body composition shown.

REFERENCES

1. Benjamin, E. *Ztschr. Kinderh.* 10: 185, 1914.
2. Wallace, W. M., Weil, W. B., Jr. and A. Taylor. *Ciba Foundation Colloquia on Ageing.* London: Churchill, 1958, vol. 4.
3. Widdowson, E. M. and R. A. McCance. *Brit. J. Nutrition* 10: 363, 1956.
4. Moulton, C. R. *J. Biol. Chem.* 57: 79, 1923.

PROTEIN REQUIREMENT IN MAN

D. Mark Hegsted

Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

Evidence on the nutritional requirements of man is derived from three sources: *a*) from epidemiologic data upon deficiency disease in relation to nutrient intakes; *b*) from specific studies with man as the subject; and *c*) from comparative studies upon other species. It will probably be generally agreed that studies with laboratory animals are the most exact but cannot be applied to man until the proper baseline or method of conversion of the data to man has been demonstrated by studies upon man himself, and that evidence from the field, while the least exact, ought to be in agreement with the more fundamental studies if they are correctly interpreted. In other words, if our estimates of the needs for any nutrient are correct or approximately so, the sum total of the evidence must fit into some logical picture. It may often be true that the apparent exceptions to a general rule or accepted thesis may provide the stimulus or approach for the correction of faulty generalizations. In this discussion on protein requirements of man, there has been no attempt to produce a complete bibliography and the references selected are, in general, only those which appear to be excellent examples of the points under discussion.

A consideration of abundant evidence upon experimental animals and upon human subjects leads to two basic considerations for the estimation of the minimum protein requirement. These have been used by the FAO Protein Committee (1) in arriving at their estimates of protein need, and a similar approach has been outlined in detail in a previous publication (2). These two underlying considerations are: *a*) the demonstration (3, 4, 5, 6) that the minimum urinary nitrogen excretion of all animals so far studied, including man, and both young and adult animals, is proportional to the basal metabolic rate and approximates 2 mg of nitrogen per basal calorie. This value is clearly not minimal in man or animals (7, 8) but may be chosen to avoid severe protein depletion as a complication; *b*) the concept of biological value (B.V.) which is a quantitative measure of the efficiency with which absorbed protein meets the body requirement for nitrogen (9, 10). A protein with a B.V. of 100 % will thus replace upon an equivalent basis the obligatory nitrogen losses in the urine. It follows, therefore, that the minimum amount of nitrogen required to maintain balance in an adult, when the dietary protein has a B.V. of 100, will be calculable from the basal metabolic rate with appropriate additions made for the usual fecal losses. The value thus obtained for an individual with a basal output of 1500 cal/day will fall at about 20 gm protein/day. Corrections may then be made for proteins of lesser B.V. If the dietary protein had a B.V. of 60%, for example, the corrected minimum need would be $20/60 \times 100 = 34.5$ gm/day. It may be emphasized that values of this order of magnitude are obtained without attempting to push the estimate to very minimal levels, the use of 1.5 mg N/basal calorie, for example, and agree with the majority of the balance studies upon adult man.

Upon a less secure basis, a similar calculation may be made for the protein requirements of growing children. The maintenance need is calculated as for the adult and to this is added the additional protein required for tissue formation (estimated from the rate of growth). This was

presumably done by the FAO Committee of Proteins (1) although the derivation of their published curve is not clear. The results of such a calculation are shown in figure 1 taken from an earlier paper (2). It is assumed that the B.V. of proteins for growth and maintenance is the same, an assumption that seems reasonable (11).

Several objections have been raised against this curve. On the one hand, it has been objected that the values are too low and obviously they are much lower than we are accustomed to think of as adequate. However, some have objected that the values obtained for the first few months of life are too high since the value approaching 2.5 gm/kg during the first months of life may exceed the amount of protein available from breast milk, especially if milk protein does not have a B.V. of 100. The problem is, then, to reconcile objections from both sides if these objections are valid.

It should be noted that such calculations emphasize the extremely slow rate of growth of children over a year or two of age and the small amount of protein, relative to the maintenance requirement, that they deposit as tissue. A 50 gm rat and a 5-year-old child may grow at about the same rate of 5 gm/day. The rapidly growing rat may deposit as much as 80% of its total protein intake as tissue and other species have much greater relative rates of growth than man and, with usual diets, much greater efficiency in deposition of body protein. In my opinion, it remains to be shown that growth in man is an important determinant in protein requirements after the first few years of life.

With respect to the first criticism that these estimates are too high for very young children, I can offer two suggestions. The intake of protein of the breast-fed infant is usually estimated by assuming that a caloric requirement of 120 cal/kg is met by the breast milk and that the milk contains approximately 1.1% protein. The recent studies of Fomon and May (12) indicate that the intake during the first months was usually between 130 and 150 cal/kg and the milk used contained about 1.2% protein. If this represents a more accurate estimate of the usual situation, then the protein supplied by breast milk will

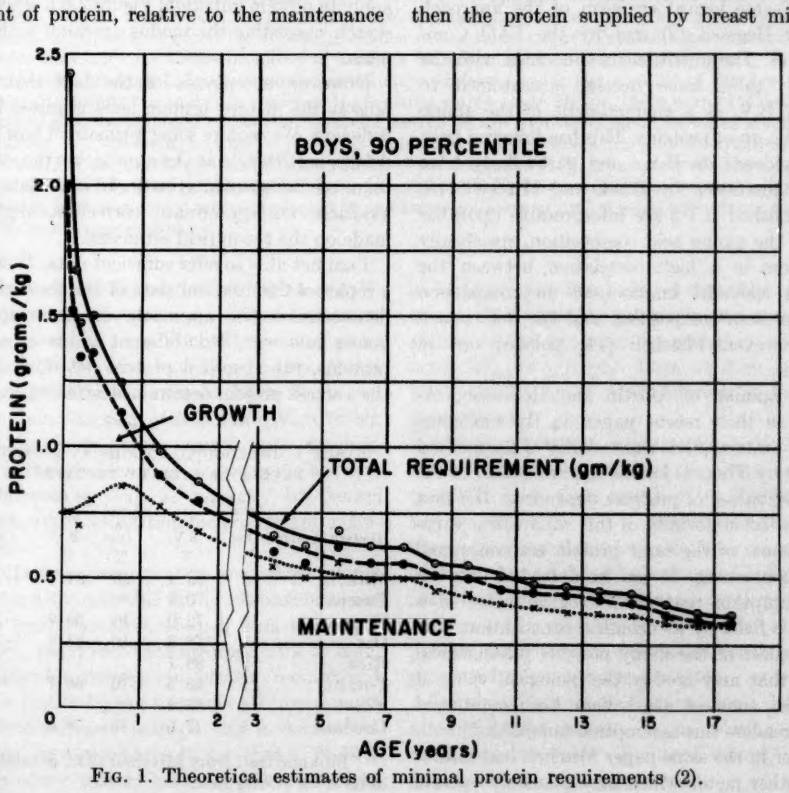


FIG. 1. Theoretical estimates of minimal protein requirements (2).

meet the theoretical estimates. It should be noted that figure 1 is based upon the growth rates of the 90 percentile (larger) boys. Secondly, the curve is based upon an estimate of 18% protein in the tissue formed. This may be slightly high at this age (12).

I am not able to find reasons for believing that theoretical baselines should be different at different ages, i.e. that there is a fundamental change in the way protein is handled by children of different ages. I conclude, therefore, that the facts as we understand them, concerning protein intakes in breast-fed children, are in essential agreement with the theoretical baselines which have been developed in studies utilizing various species. From knowledge of the performance of breast-fed infants it would follow that the protein requirement, after correction for B.V. if necessary, of young infants is not greatly different from that supplied by breast milk.

Turning now to the opposite consideration—that the values derived for older ages are too low. I am inclined to agree and here I am able to offer some logical criticism of the approach used by Hegsted (2) and by the FAO Committee (1). The nutritionists concerned with the nutritive value have become accustomed to think of B.V. as a characteristic of the amino acid make-up of proteins. This has followed from much evidence on B.V.s and particularly from the demonstration by Block and Mitchell (13) that published B.V.s are interpretable upon the basis of the amino acid composition, specifically that there is a high correlation between the 'limiting essential amino acid' in comparison to egg protein composition and the determined B.V. However, Mitchell (14) pointed out in 1923 that:

"The opinion of Martin and Robinson, expressed in their recent paper on the biological value of proteins, that the 'validity of the method adopted by Thomas for the determination of the biological values of proteins depends in the first place on the uniformity of this value when varying amounts of the same protein are consumed' is not convincing. It can be defended only on the assumption that the biological value of a protein is fixed by its chemical constitution. The total neglect of the many possible physiological factors that may modify the biological value of a protein, some of which have been mentioned above, renders this assumption untenable."

Earlier in the same paper Mitchell had said:

"Another factor which may plausibly operate in reducing the biological value of a given protein . . . as its concentration in the ration increases, is an inevitable wastage of amino acids by oxidative processes . . . It seems unreasonable to suppose that the amino acids of dietary origin are totally immune to oxidation, or are oxidized only in so far as they are not used in anabolism . . . If they are not thus totally immune to oxidation, the extent of oxidation will depend upon their concentration in the cellular fluids . . . which would in turn be largely dependent on the percentage of protein in the ration. If this wastage of amino acids by oxidation increases more rapidly than the protein intake, a greater percentage loss of absorbed nitrogen . . . will result with rations containing higher percentages of protein."

Mitchell demonstrated that the B.V.s determined in young rats decreased as the level of dietary protein was raised as is summarized in table 1. The 'law of diminishing returns' or the 'log dose-response curve' is so common in biology that there is little reason to believe it should not apply to protein nutrition. Figure 2 is a schematic sketch suggesting the modus operandi with proteins.

However, acceptance of the fact that B.V. falls as the dietary protein level is raised is not sufficient. We require some estimate of how much it falls, particularly as one approaches the amount required for optimal growth. If such data were available an appropriate correction might be made on the theoretical estimates.

I am not able to offer sufficient data. However, a replot of the excellent data of Barnes *et al.* (15) demonstrates the approach. In these studies, young rats were fed different levels of several proteins, the absorbed protein determined, and the carcass protein determined after 42 days on

TABLE 1. BIOLOGICAL VALUES AT DIFFERENT LEVELS OF DIETARY PROTEIN*

Dietary Protein	Level in Diet %	B.V. %	Level in Diet %	B.V. %	Change %
Milk	5	93.4	10	84.7	−10.3
Casein	5	70.8			
Corn	5	72.0	10	59.6	−17.2
Oat	5	78.6	10	64.9	−17.4
Rice	5	86.1			
Potato	5	68.5	10	66.7	−2.6
Yeast	5	85.5			
Coconut	5	77.0	10	58.0	−24.7

* Summarized from Mitchell (14). Studies were done with young rats.

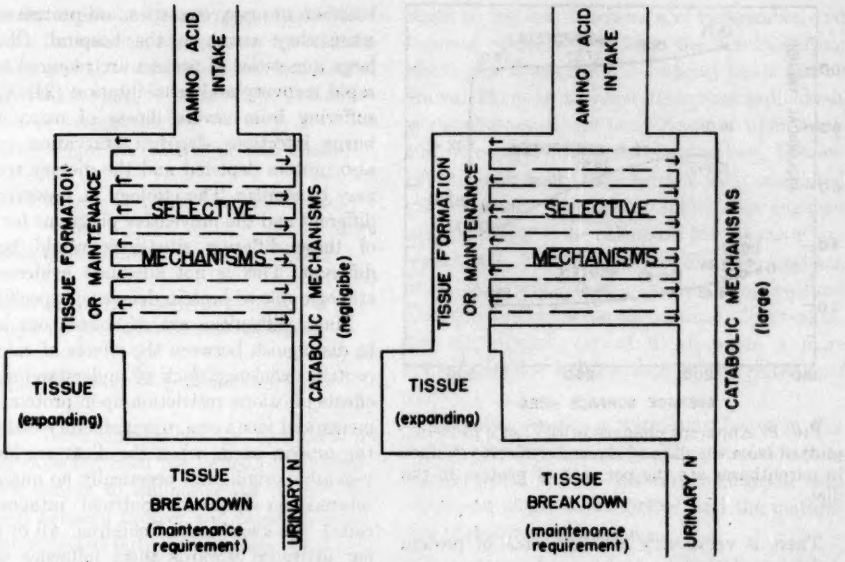


FIG. 2. The 2 animals receive sufficient amounts of protein to grow at equal and nearly maximum rates of growth, the first animal with protein of 100% B.V., and the second with protein of approximately 60% B.V. In the first animal the total protein intake is accounted for by the tissue formed and the amount of protein required for the maintenance of the body tissue. Catabolic mechanisms, other than those necessary for tissue breakdown, account for a negligible amount of the urinary excretion and are poorly developed. In the second animal approximately twice the amount of dietary protein must be fed to supply the same quantities of the limiting amino acids. Hence, there is an excess of amino acids which must be disposed of and the catabolic activities are well-developed. Selective mechanisms are, however, not sufficiently astute to direct all limiting essential amino acids into the synthetic channels and some of the limiting essential amino acids which determine B.V. are destroyed. Thus, as larger amounts of relatively poor quality proteins are fed, the net B.V. decreases. Selective mechanisms may be viewed in part as random selections based upon chance collisons of amino acids with either catabolic or synthetic enzyme systems.

experiment. The efficiency of utilization or B.V. (including both the protein required for maintenance as well as tissue deposition) can then be estimated. These values have been plotted against the average size of the animals (figure 3). As can be seen there was very little fall in B.V. with egg protein until maximum growth was reached. There is little evidence of decreasing efficiency. It is interesting that, as predicted by theory, there was 100% utilization for growth and maintenance.

With both soybean protein and wheat gluten there was a considerable fall in utilization as the dietary level increased. If such data are representative, and if such data are applicable to man, a considerable increase in protein requirement could be justified upon this basis. We need much more evidence and perhaps this is somewhere available. It seems likely to me that a 30–50% increase above current minimal estimates might be justified upon this basis. It should be clear, of course, that when the protein intake exceeds the ability of the organism to utilize it, efficiency must fall precipitously. Data at such levels of intake would be of little interest in terms of requirements.

I shall not discuss much of the balance data available upon man. Some of the pitfalls in interpretation have been outlined by Wallace (16) and by others (2, 17). I believe that there is a bias in much of the balance data for reasons not entirely clear to me. Furthermore, I believe it is clear that when dietary protein levels are raised there may be a nitrogen retention over extended periods. Such data are difficult, if not impossible, as related to protein requirements.

Turning briefly to the epidemiologic data, and attempting to consider it in relation to the basic considerations discussed above, a few points seem of some importance.

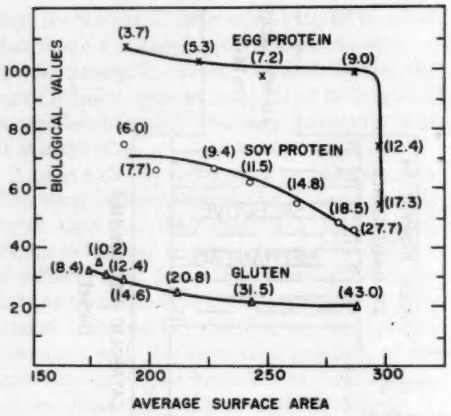


FIG. 3. Apparent changes in B.V. of 3 proteins, plotted from the data of Barnes *et al.* (15). Values in parentheses are the per cent of protein in the diet.

There is very, very little evidence of protein deficiency in adult man anywhere, including areas where diets are almost entirely of vegetable origin (18). Many vegetable diets appear capable of supporting the obviously increased protein needs of lactation (19). I consider such data as compatible with and supportive of the estimated requirements derived earlier in the paper.

I should like to recall to you the generalizations drawn about the time of World War I upon hunger edema, namely, that this was classical 'protein deficiency' and explained by the Starling hypothesis. Without denying that edema can be produced by protein deficiency, the specific role of protein in 'hunger edema' remains obscure. Caloric deficiency seems by far to be the primary determinant, its effect being modified by numerous other factors (20, 21).

I believe there is a similar tendency to oversimplify the situation in malnourished children, now usually classified as kwashiorkor or simply as 'protein malnutrition.' There is no doubt that protein deficiency can be produced in children. The disease seen in areas where the protein content of the foods is very low (sugar, cassava, etc.) (22, 23), is probably protein deficiency primarily. In general, however, investigators in this field have failed to identify the role of protein in the etiology of these conditions. Much of the current approach is entirely reminiscent of the approach to 'hunger edema' 40 years ago.

It is true that severely malnourished children, common in many countries, are protein deficient when they arrive in the hospital. Obviously, large quantities of protein are required for their rapid recovery and rehabilitation (24). Children suffering from severe illness of many kinds—burns, infections, diarrhea, starvation, etc.—are also protein depleted and the dietary treatment may be similar. The etiology is, however, much different and the preventive programs for control of these differing situations would be quite different. This is not adequate evidence of an etiologic role of protein deficiency, specifically.

Major difficulties are, of course, our inability to distinguish between the effects of caloric and protein deficiency, lack of understanding of the effects of caloric restriction upon protein requirements and vice versa, unsatisfactory estimates of the protein needs when the diets are largely of vegetable origin, and practically no quantitative information upon the nutrient intakes of so-called 'pre-kwashiorkor' children. All of these or our attitudes towards them influence our conclusions.

Information upon the food habits of the age group of interest are extremely difficult to obtain. The only data of the kind desired that I am aware of were briefly reported by Scrimshaw (25). These are only 3 day intakes on a series of 15 children and I am aware that I am greatly overworking the data. I do so only for illustrative purposes. I arranged the data upon calorie intake

TABLE 2. RECENT ESTIMATES OF PROTEIN NEEDS

Age	F.A.O.[1]	Princeton Conference[2]	Theoretical Minimum[3]	Estimated Need With B.V. = 70[4]
	gm/kg	gm/kg	gm/kg	gm/kg
First month	2.1	2.2–2.5	2.4	[5]
2–5 months	1.8	2.0	1.5	[5]
6–12 months	1.4	1.5–2.0	1.25	2.36
1–5 yr.	1.0	1.5	0.7	1.3
5 yr.–adolescence	.75	1.5	0.6	1.1
Adolescence	.9	over 1.5	0.5	1.0
Adults	.35	0.5	0.35	.65

[1] Values assume B.V. of 100 to be corrected according to B.V. of diet (1).

[2] Apparently assumes dietary protein of reasonably good quality (25).

[3] Protein of B.V. = 100 (2).

[4] Including correction for B.V., i.e. 100/70, as well as 30% additional for fall in B.V.

[5] Milk diet, no correction.

and protein intake as percentages of the NRC Allowances for calories, and protein for children of this size, and the body weights as per cent of the 50 percentile chart of Stuart (26). The correlations found were as follows:

r_{12} (relative weight vs. relative protein intake) $= 0.05$

r_{13} (relative weight vs. relative caloric intake) $= 0.22$

r_{23} (relative protein intake vs. caloric intake) $= 0.50$

The partial correlations were as follows:

$r_{12.3}$ (weight vs. protein—calories constant) $= -0.09$

$r_{13.2}$ (weight vs. calories—proteins constant) $= 0.32$

None of these correlations are significant as might be expected from the limited data. I present them simply to emphasize the rather high correlation between protein and calorie intakes. These are expected but often ignored. Also, that as far as these data go, the indications are that the caloric lack may be more important relative to physical status than the protein lack. Of course, a protein deficiency will automatically limit the calorie intake, starting the familiar vicious cycle. My concern is with what starts it. We may recall that Macy and Hunscher (27) concluded that a difference of intake of 10 cal/kg/day may spell success or failure in children.

Finally, as attested by hundreds of experiments, a diet somewhat limited in protein fed to young, growing animals of various kinds limits growth but produces a rather benign deficiency. I am told that 'sugar babies' which may be the prototypes of true protein deficiency respond rapidly to treatment.

Although the Princeton Conference did not propose official allowances, the sense of the meeting is summarized in table 2, together with minimal estimates taken from figure 1 and correction of these values for change in B.V. as suggested earlier in the text. The estimates of this group correspond to theoretical calculation reasonably well for infants and adults and less well for children and adolescents. More work and evidence is clearly needed in this area.

The emphasis in this talk has been upon minimal needs. This is because the minimal needs ought to be, and I believe are, measurable, and minimal values should form the baselines from which logical estimates of optimal needs can be drawn. There is no implication that individuals or populations should be maintained upon minimal or marginal intakes. Nutritionists, like engineers must build their bridges with calculated tolerances and margins of safety. An engineer will 'go broke' if he calculates his tolerance unrealistically. Nutritionists face the same problem in countries which lack economic or agricultural resources to meet recommendations. This realization has already served to stimulate a more critical attitude toward some of the nutritional dicta.

Resources are not a restraining factor in this country; in fact, the reverse. The restraint is coming, however, from a growing realization that 'more and bigger is not better' and the maturation of nutrition as a science.

REFERENCES

1. *Report of the FAO Committee "Protein Requirements."* Food and Agriculture Organization of the United Nations, Rome: 1957.
2. HEGSTED, D. M. *J. Am. Dietet. A.* 33: 225, 1957.
3. TERROINE, E. F. *Quart. Bull. Health Organisation League Nations* 5: 427, 1936.
4. SORG-MATTER, H. *Arch. internat. physiol.* 30: 126, 1928.
5. SMUTS, D. B. *J. Nutrition* 9: 403, 1935.
6. BRODY, S. *Bioenergetics and Growth.* New York: Reinhold, 1945.
7. BRICKER, M., H. H. MITCHELL AND G. M. KINSMAN. *J. Nutrition* 30: 269, 1945.
8. ASHWORTH, U. S. AND S. BRODY. *Univ. Mo. Agr. Exp. Sta. Res. Bull.* 189 and 190, 1933, and 223 and 228, 1935.
9. MITCHELL, H. H. *J. Biol. Chem.* 58: 873, 1923.
10. ALLISON, J. B. *Physiol. Rev.* 35: 664, 1955.
11. MITCHELL, H. H. *Protein and Amino Acid Requirements of Mammals*, edited by A. A. ALBANESE. New York: Acad. Press, 1950, p. 1.
12. FOMAN, S. J. AND C. D. MAY. *Pediatrics* 22: 101, 1958.
13. BLOCK, J. J. AND H. H. MITCHELL. *Nutr. Abst. and Rev.* 16: 249, 1946.
14. MITCHELL, H. H. *J. Biol. Chem.* [illegible]: 905, 1923.
15. BARNES, R. H., M. J. BATES AND J. E. MAACK. *J. Nutrition* 32: 535, 1946.
16. WALLACE, W. M. *Fed. Proc.* 18: 1125, 1959.
17. HEGSTED, D. M. *Methods for Evaluation of Nutritional Adequacy and Status.* Washington: Office of Quartermaster General, 1954, p. 103.
18. PATWARDHAN, V. N. *Voeding* 16: 223, 1955.

19. WALKER, A. R. F. *Ann. New York Acad. Sc.* 69: 989, 1958.
20. KEYS, H., J. BROZEK, A. HENSCHEL, O. MICKELSON, AND H. L. TAYLOR. *The Biology of Human Starvation*. Minneapolis: University of Minnesota Press, 1950.
21. MEDICAL RESEARCH COUNCIL. *Special Rept. Ser. No. 275*. London: His Majesty's Stationery Office, 1951.
22. JELLIFFE, D. B. *Infant Nutrition in the Subtropics and Tropics*. Geneva: World Health Organ. 1955.
23. *FAO Protein Malnutrition* (Proceedings of a Conference in Jamaica). New York: Macy, 1955.
24. TROWELL, H. C., J. N. P. DAVIES AND R. F. A. DEAN. *Kwashiokor*. London: Arnold, 1954.
25. SCRIMSHAW, N. S. *Human Protein Requirements and their Fulfillment in Practice*, edited by J. C. WATERLOW and J. M. L. STEPHAN, FAO, Rome, 1957, p. 26.
26. STUART, H. C. *Percentile Charts of Measurements, Boys and Girls*. Boston: Childrens Medical Center.
27. MACY, I. G. AND H. A. HUNSCHER. *J. Nutrition* 45: 189, 1951.

AMINO ACID BALANCE AND PROTEIN REQUIREMENT

A. E. HARPER AND U. S. KUMTA

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

IN THE LIGHT of our present knowledge of amino acid requirements the term 'protein requirement' may be considered vague and outmoded. Living organisms certainly do not require protein as such. They require, instead, nitrogen and, for most species, a few specific organic molecules. The organic molecules can, in a number of cases, be provided as keto- or hydroxy-organic acids, and the nitrogen as an ammonium salt. Therefore, the actual requirements that are satisfied by the ingestion of protein are those for nitrogen, for certain organic acids and for a few amino acids that the body is unable to synthesize or cannot synthesize in adequate quantity. Further, since different proteins contain different quantities of the amino acids required by living organisms, protein requirements can be expressed only in terms of specific proteins or protein mixtures. Methods for comparing the biological values of food proteins are, therefore, of great practical importance. It is recognition of these facts that has led to the concept of amino acid balance, a concept that provides a sound fundamental basis for estimating the biological value of a protein from a knowledge of its amino acid composition.

The concept of amino acid balance is not new. The observations of Willcock and Hopkins (1) and Osborne and Mendel (2), that certain amino acids were essential nutrients and that proteins differed in their content of these amino acids, led them to suggest that the nutritive value of a protein depended upon its amino acid composition. The concept was developed by Block and Mitchell (3) who showed that a fairly good estimate of the biological value of a protein can be obtained from a comparison of its amino acid composition with that of whole egg proteins. They calculated which amino acid was in greatest deficit with respect to whole egg proteins and demonstrated that there was a high correlation between the magnitude of the greatest deficit for a single indispensable amino acid, expressed as a percentage, and the biological value of the protein as determined experimentally. Oser (4) suggested using the geometric mean of all the amino acid deficits as an alternative method of calculating the nutritive value of a protein. The values obtained by this method showed almost the same relationship to biological value as those obtained by the method of Block and Mitchell. Almquist (5) has discussed, more particularly, the importance of amino acid balance as a factor influencing the efficiency of protein utilization and has elaborated certain principles implicit in such a concept.

The essence of these principles is that the efficiency with which a protein can be used as a source of amino acids for tissue synthesis depends very largely upon how closely the relative proportions of the indispensable amino acids it contains coincide with the relative proportions in which the amino acids are required by the body. Proteins such as those of whole egg, which approach most closely to the ideal (3) are termed well-balanced proteins. Proteins which

deviate from the ideal are termed unbalanced. However, there are many degrees of deviation, ranging from casein, which is slightly low in the sulfur-containing amino acids, to zein and gelatin, which completely lack an essential amino acid.

This concept has been modified by Flodin (6) who calculated the relative proportions of the various essential amino acids in different proteins by setting the tryptophan content in each case at one. The sets of proportions obtained in this way can be compared simply and graphically. It should be emphasized, however, that the balance concept is something more than just a mathematical relationship. It is clear from the papers of Block and Mitchell (3), Oser (4) and Almquist (5) that it must have a firm quantitative base. This base may be the individual amino acid requirements (gm/kg of body weight, gm/100 gm of diet, gm/day) or the percentage composition of a mixture of proteins of known high biological value (such as those of whole egg). These are concrete numbers, they are not the abstract numbers of a proportion.

Starting from this point amino acid balance can be related in two ways to protein requirements. In the first place, the amino acid balance of a protein determines very largely, as was mentioned above, the efficiency with which the protein can be used as a source of amino acids by the body and, therefore, how much of that particular protein must be fed to meet the amino acid requirements. In the second place, the amino acid balance of a diet may be so poor that the performance of subjects consuming the diet falls below that expected from a knowledge of the amino acid composition of the dietary protein. This condition, which is described as an amino acid imbalance, causes an increase in amino acid requirements expressed as a percentage of the diet (7, 8).

Amino acid balance and efficiency of protein utilization. In a discussion of the protein requirements of man the relationship between amino acid balance and efficiency of protein utilization is the more important. If a protein provides all of the amino acids in exactly the proportions in which they are required by the body, it will be used very efficiently. However, if only the relative proportions of the indispensable amino acids are considered, the quantitative aspects of amino acid balance may be obscured.

A consideration of quantitative relationships makes it clear that two proteins may contain exactly the same proportions of the indispensable amino acids, yet be used with distinctly different efficiencies. Whole egg proteins, for example, contain roughly 60% of the indispensable and semi-indispensable amino acids, whereas, the proteins of whole wheat contain just over 40% of these amino acids. Therefore, even if whole wheat is supplemented with the most limiting amino acid, lysine, to bring its amino acid balance very closely into line with that of whole egg proteins or the amino acid requirements, it will still provide considerably less of several of the indispensable amino acids per unit of nitrogen than do whole egg proteins (compare fig. 1*A* and *B*). Or in other words, when these two protein mixtures are fed in equal quantities, even if wheat proteins are supplemented with lysine so that the relative proportions of the indispensable amino acids in both may be quite similar (fig. 1*B*), they will be used with distinctly different efficiencies. This, of course, is because whole egg proteins contain 1.6% of tryptophan while wheat gluten contains 1.2% (9), and if these are both set at 1 in calculating the relative proportions of the other amino acids (fig. 1*B*), the

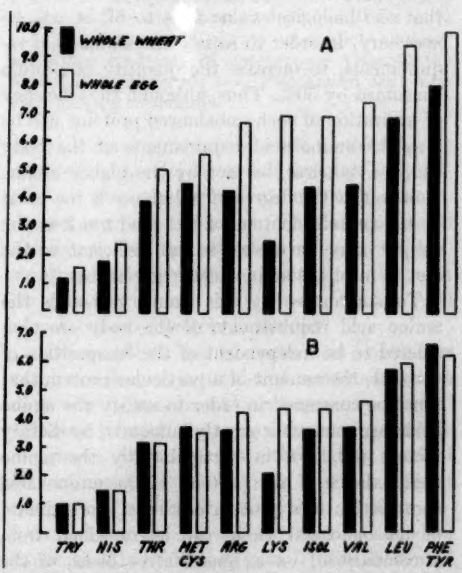


FIG. 1. *A*: Amino acid composition (%) of whole wheat and whole egg proteins (16% nitrogen basis). *B*: Proportions of the indispensable amino acids in whole wheat and whole egg proteins (tryptophan =1).

quantitative differences between the two proteins are obscured. It is presumably this relationship which accounts for the observation of Bender (10) that lysine supplementation is of only limited value in improving the biological value of wheat proteins.

It should also be noted that in the attempt of the FAO Committee on Protein Requirements to devise an ideal pattern of amino acids to serve as a base for comparing the nutritive values of food proteins, the pattern presented has a firm quantitative basis (mg amino acid per gm of nitrogen) (11). If, in the pattern for a food protein calculated on this basis, the value for any one of the indispensable amino acids is lower than that in the ideal pattern, the efficiency of utilization of the food protein will be low in proportion to the magnitude of the deficit. The effect of this lack of a sufficient quantity of an amino acid can be alleviated in either of two ways. First, by supplementing the diet or the foodstuff with well-balanced protein or with the specific amino acid which is in low amount; second, by increasing the intake of the unbalanced protein (5).

In using the second procedure, if we assume that the relative proportions of the amino acids in the proteins of a diet deviate from the ideal so that the biological value falls to 67, it will be necessary, in order to satisfy the amino acid requirements, to increase the quantity of protein consumed by 50%. Thus, although the efficiency of utilization of such unbalanced proteins will be low, the amino acid requirements of the body can, nevertheless, be met by the higher intake. However, if the degree of unbalance is too great or the protein content of the food too low, the subject may be unable to eat sufficient of the diet to meet the amino acid requirements.

To summarize at this point, although the amino acid requirements of the body are considered to be independent of the composition of the diet, the amount of a particular protein that must be consumed in order to satisfy the amino acid requirements is greatly influenced by dietary factors (12, 13), in particular, by the amino acid balance of the protein. If the amino acid composition of a protein is known, an estimate of its biological value can be obtained from a comparison, on a quantitative basis, of the proportions of the various amino acids it contains with the proportions of the requirements of the body or with the proportions of the indispensable amino acids in a protein of known high biological value.

Amino acid imbalance. Secondly, protein requirements or amino acid requirements may be influenced by the occurrence of amino acid imbalances (7, 8). Although such effects can be discussed independently of the influence of amino acid balance on the efficiency of protein utilization, there is a distinct relationship between them. Amino acid imbalances occur when the percentage of one (or more) of the amino acids in a diet is so low that, not only does the efficiency of protein utilization fall, but some additional adverse effect, such as a drop in food consumption, a depression in growth or an increase in the need for one or more amino acids becomes evident. Almost all of the information about amino acid imbalances has been obtained through animal experiments so it is difficult to assess its significance in human nutrition. However, as amino acid imbalances have been demonstrated in experiments involving several species of animals, discussion of the relationship between amino acid imbalance and protein requirements needs no justification.

At this point, it should be made clear that amino acid imbalances can be distinguished from certain other cases in which adverse effects are caused by excesses of amino acids (7). Excessive intakes of certain individual amino acids, such as methionine or tyrosine, appear to cause definite toxic reactions. These seem to be highly specific and to give rise to symptoms which cannot be prevented by adding a small quantity of the most limiting amino acids to the diet (7, 14).

Another case has been described as an amino acid antagonism due to an excess of leucine in the diet. This becomes evident through growth retardation. The signs are prevented by adding the structurally similar amino acid, isoleucine, along with leucine; but again, not when a supplement of the most limiting amino acid in the diet is provided (14).

In contrast to these, the adverse effects caused by amino acid imbalances are prevented when a quantity, usually a relatively small quantity, of the indispensable amino acid (or acids) that is most limiting for growth is added to the diet. In other words, an amino acid imbalance increases the need (expressed as a percentage of the diet) for the amino acid that is most limiting for growth.

It might also be well to distinguish between the terms 'imbalanced' and 'unbalanced'. A protein that is low in one or more of the essential amino acids is an unbalanced protein. Through-

out the literature, one can find such proteins described as either unbalanced or imbalanced. There is less danger of confusion if the latter term is used ony to describe the situation in which the addition of a quantity of an unbalanced protein, or amino acid mixture, to a diet causes a growth retardation or some other adverse effect which can be prevented by a supplement of the amino acid that is most limiting in the diet for normal body function.

An amino acid imbalance, as it has been defined above (7), was first clearly demonstrated in experiments on niacin deficiency in rats when it was observed that the addition of gelatin, or even of threonine alone, to the low protein diet used in those studies caused a severe growth depression (15). It has since become clear that this effect is only indirectly related to the niacin content of the diet and depends primarily upon the relative proportions of the indispensable amino acids in the diet (7, 8). The results of an experiment on rats illustrating this are presented in table 1. The control group was fed a diet containing 8% of casein, but adequate in all other respects. In the diets of the other groups a portion of the carbohydrate (autoclaved starch) was replaced by gelatin. As the level of gelatin was increased, or in other words, as the dietary protein became more unbalanced, growth was retarded, even though in each case the diet contained the same percentage of casein (16). The growth retardation was prevented by a supplement of tryptophan, the amino acid most limiting for growth.

The extent to which a given level of unbalanced protein will retard growth depends upon the level of reasonably well-balanced protein in the diet (16). This is illustrated in figure 2. The curves represent the rates of gain of groups of rats fed on diets containing various levels of casein and 0.2% of DL-methionine with or without a supplement of all of the essential amino acids except threonine, in the quantities in which they are present in 6% of casein. The amino acid mixture lacking threonine caused a growth depression, the greatest depression occurring when the diet contained an intermediate level of casein. The growth depression was smaller when the dietary level of protein was lower or higher but, if the growth rates of the retarded groups are expressed as percentages of their respective control groups, the growth depression is seen to become less severe as the casein content of the diet is increased (16). When the dietary level of casein was between 12 and 15% (approximately sufficient, when supplemented with methionine, to support the maximum rate of growth), the addition of the unbalanced amino acid mixture caused no growth depression. Thus an amino acid imbalance can be prevented either by supplementing the diet with a quantity of the most limiting amino acid or by increasing the level of well-balanced protein in the diet.

TABLE 1. AMINO ACID IMBALANCE CREATED BY ADDING INCREMENTS OF GELATIN TO A DIET CONTAINING ONLY 8% OF CASEIN AND 0.2% OF DL-METHIONINE AS THE SOURCE OF PROTEIN BUT ADEQUATE IN ALL OTHER RESPECTS

Diet Composition	Wt. Gain (2 Weeks)
	gm
Basal diet (8% casein + 0.2% DL-methionine)	54±4
+6% gelatin	52±3
+9% gelatin	40±5
+12% gelatin	34±5
+15% gelatin	24±3
+15% gelatin + 0.2% DL-tryptophan	77±5

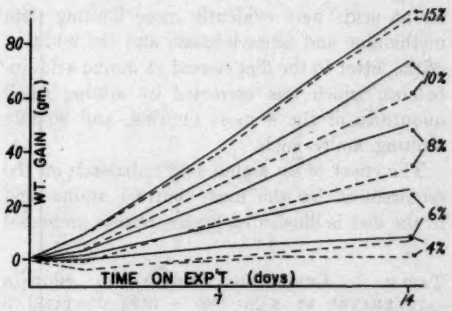


FIG. 2. Effect of a mixture of indispensable amino acids lacking threonine (in quantities =6% casein) on the growth of rats fed on diets containing different levels of casein; —— Control (no amino acid mixture); ----- Plus amino acid mixture lacking threonine.

Adverse effects due to amino acid imbalances can, nevertheless, be produced in animals fed on diets that contain a sufficient quantity of well-balanced protein to support the maximum rate of growth. However, when the dietary level of well-balanced protein is high a much greater quantity of an unbalanced protein or of an unbalanced amino acid mixture must be added to cause a fall in the rate of gain. This is illustrated by the results in table 2 which show that a supplement of between 12 and 15% of gelatin was required to cause a retardation in the growth of rats receiving sufficient casein to support close to maximum growth in the absence of the gelatin.

Amino acid imbalances caused by the addition of relatively small quantities of a few amino acids to low protein diets are sometimes observed in experiments on either young growing animals or mature animals (7, 17). Such an effect is illustrated in table 3. In this case the diet contained 6% of fibrin, sufficient to support a rate of growth of about 15 gm/wk. The addition to this diet of methionine and phenylalanine, which were calculated to be the most limiting amino acids, caused a severe growth depression. Only when a mixture of the 4 amino acids—histidine, leucine, isoleucine, and valine—was added was the growth retardation prevented. The latter 4 amino acids were evidently more limiting than methionine and phenylalanine and the addition of the latter to the diet caused an amino acid imbalance which was corrected by adding small quantities of the 4 most limiting, and equally limiting, amino acids.

The effect of an amino acid imbalance on the requirement for the most limiting amino acid in the diet is illustrated by the results presented in table 4. In this case, in order to study the requirement for a single amino acid, young rats were fed a diet containing 6% of fibrin and a mixture of amino acids lacking only histidine. The mixture lacking histidine caused a depression in food intake and a severe growth retardation. The subsequent addition of histidine stimulated food intake and prevented the growth retardation. As can be seen from the table, in order to accomplish this, the level of histidine in the diet had to be increased by approximately 0.05%, it may also be noted however, that the total histidine intake per unit of gain was higher than that of the control group when the growth depression was prevented.

Nitrogen balance studies were carried out in an attempt to determine whether nitrogen retention was influenced by an amino acid imbalance (17, 18). Mature protein-depleted rats were used. The control diet, which contained 6% of fibrin, was made isonitrogenous with the diet containing methionine and phenylalanine. The results presented in table 5 show that the growth of the group fed the diet containing fibrin and additional methionine and phenylalanine was retarded, and that these animals retained a smaller percentage of ingested nitrogen than the control rats fed *ad libitum*. In order to assess the effect of the drop in food intake caused by the imbalance a pair-fed control group was included. The growth of the pair-fed control animals was as low as that of the animals consuming the imbalanced diet and the value for the percentage of ingested nitrogen retained by this group was even lower than that for the group fed the imbalanced diet.

TABLE 2. LEVEL OF UNBALANCED PROTEIN TOLERATED BY RATS FED A DIET CONTAINING 12% OF CASEIN SUPPLEMENTED WITH 0.3% OF DL-METHIONINE

Diet Composition	Wt. Gain (2 Weeks)
	gm
Basal diet (12% casein + 0.3% DL-methionine)	75±2
+6% gelatin	81±2
+9% gelatin	80±3
+12% gelatin	78±2
+15% gelatin	70±4
+15% gelatin + 0.3% DL-tryptophan	83±4

TABLE 3. AMINO ACID IMBALANCE-INDUCED DEPRESSION IN GAIN IN WEIGHT AND ITS CORRECTION BY LIMITING AMINO ACID(S)

Diet		Wt. Gain (2 Weeks)
		gm
I. 6% Fibrin		33±2.5
II. 6% Fibrin	+0.4% DL-methionine +0.6% DL-phenylalanine	19.2±2.3
III. Diet II	+0.05% L-histidine HCl +0.1% L-leucine +0.1% DL-isoleucine +0.15% DL-valine	44.5±1.2

TABLE 4. INFLUENCE OF AMINO ACID IMBALANCE ON THE INCREASED NEED FOR THE LIMITING AMINO ACID

Diet	Wt. Gain (14 Days)	Diet Consumed	Histidine Consumed
	gm	*gm*	*gm*
I. 6% Fibrin	32.2	620	0.99
II. 6% Fibrin + amino acid mixture lacking histidine*	2.2	186	0.29
III. Diet II + 0.05% L-histidine	32.8	575	1.21

* Amino acid mixture contained DL-methionine 0.4%; DL-phenylalanine 0.6%; L-leucine 0.4%; DL-isoleucine 0.4%; DL-valine 0.7%; L-lysine·HCl 0.6%; L-arginine HCl 0.2%; DL-tryptophan 0.2%; DL-threonine 0.4%; L-glutamic acid 1%.

These observations indicated that an amino acid imbalance caused a fairly rapid fall in food intake and that this influenced nitrogen retention sufficiently to prevent any specific effect of the imbalance from being detected. In an attempt to overcome this problem rats were trained to consume their entire daily ration in 2 hours on the assumption that this regimen would provide a method of increasing the food intake of the group fed the imbalanced diet and that this would exaggerate any effect of the imbalance on nitrogen retention. However, even under these conditions, food intake dropped after the first feeding (18).

In order to study in more detail the effect of amino acid imbalance on food intake, a large number of protein-depleted rats were separated into 2 groups, one of which was allowed to eat *ad libitum* the diet containing 6% of fibrin; the other, the same diet to which had been added sufficient methionine and phenylalanine to create an imbalance. Food consumption records were taken at short intervals, and the results obtained are shown in figure 3. The two groups consumed their respective diets at approximately the same rapid rate for about 3 hours, then, the food intake of the rats consuming the imbalanced diet fell off sharply. When, to the imbalanced diet a mixture of histidine, leucine, isoleucine and valine was added, the rats resumed eating rapidly within 8 hours. Evidently food intake was affected within a few hours by the creation of an amino acid imbalance indicating that the initial effects of amino acid imbalances must be studied within this time.

It was surprising how rapidly and how severely food intake was depressed by the addition of between 0.5% and 1.0% of the mixture of methionine and phenylalanine and how effectively food consumption and growth were stimulated when the mixture of histidine, leucine, isoleucine and valine was provided The effect of the imbalance was to increase the requirements, expressed as a percentage of the diet, for these 4 amino acids. In the case in which a diet low in histidine was studied (table 4) the actual intake, as well as the percentage in the diet, was higher when the growth retardation due to the unbalanced amino acid mixture was prevented These observations suggest that there is a decrease in the efficiency of utilization of the most limiting amino acid in the diet as a result of an amino acid imbalance.

It is easy to envision that amino acid catabolism may be stimulated when a large quantity of an unbalanced protein is added to a diet and that some of the most limiting amino acid may be destroyed (19). Such an effect, like any amino acid deficiency, would result in a fall in food consumption and in the need for an increased intake

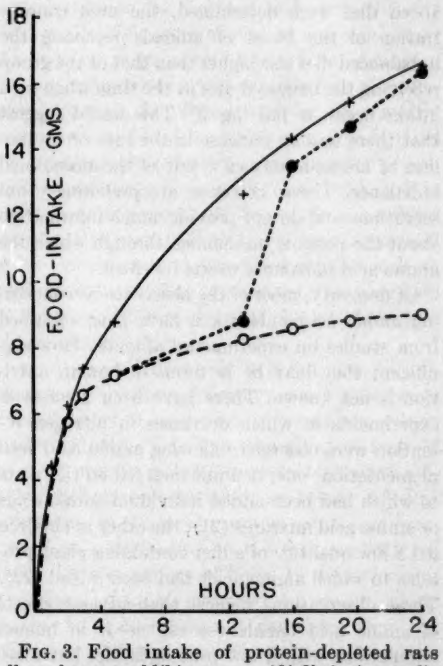


FIG. 3. Food intake of protein-depleted rats allowed to eat *ad libitum*; —— 6% fibrin (control); ----- 6% fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine, (imbalance); ····· Imbalance corrected by adding leucine, isoleucine, valine and histidine.

TABLE 5. EFFECT OF AMINO ACID IMBALANCE ON NITROGEN BALANCE OF PROTEIN DEPLETED ADULT RAT

Diet	Nitrogen Intake*	Wt. Change	Nitrogen Retained
	mg	*gm*	%
6% Fibrin (control)	483.0±6†	19.2±3.8	59.9±1.1
6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	227.8±5.7	3.2±2.1	43.7±1.5
Pair-fed control	227.8±5.7	2.3±2.1	32.9±5.0

All diets made iso-nitrogenous with L-glutamic acid.
* 3-day experimental period. † S. E. of mean for 6 rats.

of the most limiting amino acid. A direct inhibition of protein synthesis would have much the same effect, so would competition in amino acid transport mechanisms.

In an effort to determine whether protein synthesis might be directly inhibited, the influence of the ingestion of the imbalanced diet on the capacity of protein-depleted rats to synthesize tryptophan-peroxidase in response to an injection of tryptophan (20) was studied. No evidence that the imbalance interfered with the synthesis of this enzyme in the liver was obtained.

However, when blood urea levels of protein-depleted rats fed the control diet or the imbalanced diet were determined, the urea concentration of the blood of animals receiving the imbalanced diet was higher than that of the group receiving the balanced diet at the time when food intake began to fall (fig. 3). This would suggest that there was an increase in the rate of catabolism of amino acids as a result of the amino acid imbalance. These, however, are preliminary observations and do not provide much information about the possible mechanism through which the amino acid imbalance exerts its effect.

Of necessity, most of the observations concerning amino acid imbalances have been obtained from studies on experimental animals. How significant they may be in terms of human nutrition is not known. There have been 2 series of experiments in which decreases in nitrogen retention were observed following amino acid supplementation: one, in adult men fed on rice diets to which had been added individual amino acids or amino acid mixtures (21); the other in children fed a low quantity of a diet containing plant proteins to which amino acids had been added (22). These observations suggest that adverse effects of amino acid imbalances can occur in human populations with a low protein intake but more evidence is essential before the significance of such effects can be appraised.

The results of animal experiments indicate that the addition of a quantity of an unbalanced protein or amino acid mixture to a diet that is low in protein will cause a severe depression in food intake and an increased need for the most limiting amino acid or acids in the diet. Whether such conditions are encountered in areas where protein intakes are low is not known but the possibility that the consumption of poorly balanced proteins contributes to the development of Kwashiorkor with its resultant depression in food intake makes an interesting speculation. It appears improbable, however, that the protein or amino acid requirements of a population having a high average protein intake are influenced by amino acid imbalances.

In summary, then, the efficiency of utilization of a protein, and therefore the amount needed to satisfy the amino acid requirements of the body, depends upon the actual quantities of the indispensable amino acids it contains as well as upon the proportions in which they occur. Proteins having the same proportions of the indispensable amino acids will be used with different efficiencies if they contain different quantities of the indispensable amino acids.

Also, the amino acid balance of a diet can deviate sufficiently from the ideal to cause a depression in food intake and in growth. This is readily demonstrated in experimental animals consuming a low protein diet and results in an increase in the requirements for the most limiting amino acids.

REFERENCES

1. Willcock, E. G. and F. G. Hopkins. *J. Physiol.* 35: 88, 1906.
2. Osborne, T. B. and L. B. Mendel. *J. Biol. Chem.* 17: 325, 1914, and 20: 351, 1915.
3. Block, R. J. and H. H. Mitchell. *Nutrition Abstr. & Rev.* 16: 249, 1946.
4. Oser, B. L. *J. Am. Dietet. A.* 27: 396, 1951.
5. Almquist, H. J. *Arch. Biochem.* 46: 250, 1953, and 52: 197, 1954.
6. Flodin, N. W. *J. Agr. Food Chem.* 1: 222, 1953.
7. Harper, A. E. Ann. New York Acad. Sc. 69: 1025, 1958.
8. Salmon, W. D. *Am. J. Clin. Nutrition* 6: 487, 1958.
9. Block, R. J. and K. W. Weiss. *Amino Acid Handbook.* Springfield, Ill.: Thomas, 1956.
10. Bender, A. E. *Science* 127: 874, 1958.
11. *FAO Nutritional Studies*, No. 16. Organization of the United Nations, Rome: Food and Agriculture, 1957, pp. 24–40.
12. Allison, J. B. *Physiol. Rev.* 35: 664, 1955.
13. Harper, A. E. Fed. Proc. 18: 104, 1959.
14. Harper, A. E., D. A. Benton and C. A. Elvehjem. *Arch. Biochem.* 57: 1, 1955.
15. Elvehjem, C. A. and W. A. Krehl. *Bordens Rev. Nutr. Res.* 16: 69, 1955.
16. Harper, A. E. *J. Nutrition.* In press.
17. Deshpande, P. D., A. E. Harper and C. A. Elvehjem. *J. Biol. Chem.* 230: 327 and 335, 1958.
18. Kumta, U. S., A. E. Harper and C. A. Elvehjem. *J. Biol. Chem.* 233: 1505, 1958.
19. Salmon, W. D. *Arch. Biochem.* 51: 30, 1954.
20. Knox, W. E. and V. H. Auerbach. *J. Biol. Chem.* 214: 307, 1955.
21. Hundley, J. M., H. R. Sandstead, A. G. Sampson, G. O. Whedon, H. Bakerman and R. Ing. *Am. J. Clin. Nutrition* 5: 316, 1957.
22. Bressani, R., N. S. Scrimshaw, M. Behar and F. Viteri. *J. Nutrition* 66: 591, 1958.

PROTEIN NUTRITION AND ENZYME CHANGES IN MAN

J. C. WATERLOW

Medical Research Council, Tropical Metabolism Research Unit, University College of the West Indies, Jamaica, West Indies

ANIMAL EXPERIMENTS and studies on human beings have shown that the protein content of the diet may affect the activity of enzymes in the body in several different ways. These observations give rise to two questions. The first is practical—how far can changes in enzyme activity be used to diagnose or assess the state of protein nutrition? The second is theoretical—what light do these changes throw on the mechanisms of protein metabolism and their relation to protein intake? This article is concerned mainly with the practical question, as it applies to man.

It is, of course, untenable in theory and unrealistic in practice to treat the metabolism of one foodstuff—protein—or the effects of its deficiency as if they occurred in isolation, uninfluenced by other nutrients. It is a rule, which like all rules has its exceptions, that deficiency states in man tend to be multiple. Human diets that are low in protein are often low in vitamins, particularly of the B complex, and in minerals, such as potassium. Again, the close relation between nitrogen and carbohydrate metabolism and between the intake of protein and of calories has been recognized for a century. Recent work on these problems has been ably reviewed by Munro (1). In spite of the experimental work, it is still difficult to disentangle the effects of these variables in man. For instance, it is not known whether famine edema is caused by deficiency of protein or of calories or of both. Another relevant example is the fatty liver of kwashiorkor (2); it is thought that this results from a relative excess of carbohydrate in a protein-poor diet, but the evidence is only circumstantial (3, 4).

In this article, therefore, the terms protein malnutrition, protein deficiency or protein depletion are used in a general sense, and not to indicate a pure deficiency of one single foodstuff. Rather they suggest a theme on which there may be many variations. The three terms, although all general, are not synonymous (5). 'Protein deficiency' is a process; 'protein depletion' is the state produced by that process. 'Protein malnutrition' also refers to a process, and although less familiar, is a more accurate term than protein deficiency. It takes account of two things—the fact, emphasized above, that the process may be complex, and the fact that a diet may be unsatisfactory not because its protein is insufficient, but because it is unbalanced (6).

Experimental studies in animals on enzyme changes in protein depletion have recently been summarized in an excellent and comprehensive review (7). This work should be complementary to that on man, since it has been concerned mainly with tissue enzymes, whereas measurements in man, for obvious reasons, have mostly been done on blood or digestive juices. It must be admitted, that the very large number of animal experiments that have been published in recent years present on the whole a confused picture. However, from this body of experimental work certain general principles can be derived, and cautionary points emphasized, that may help in the interpretation of results obtained in human protein malnutrition.

a) Even experimentally it is not easy to produce a pure protein deficiency, since progressive loss of appetite may lead to secondary caloric undernutrition. Yet, from the point of view of over-all protein metabolism, uncomplicated protein deficiency and starvation are totally different situations. In the former protein catabolism is reduced to a minimum—to the endogenous level, in the old terminology of Folin. In the latter protein catabolism is increased to cover caloric needs. It is therefore not surprising that the effects on enzyme systems are not always the same. This complicates the interpretation of measurements in man, in whom deficiencies of protein and of calories usually coexist to a greater or lesser degree.

b) Very often the conclusions that can be drawn depend on the basis of reference that is chosen. For example, on a given diet there may be no change in the concentration of an enzyme —i.e. its activity per unit weight or unit protein or unit nitrogen; but there may be a large fall in the total amount of enzyme in an organ such as the liver, because that organ is reduced in size. When it is impossible to measure the mass of an organ, as in the case of muscle, or of human tissue specimens taken by biopsy, the best

standard of reference is probably DNA (deoxyribose nucleic acid). This gives a measure of the enzyme activity per cell, although the measure is necessarily approximate, because no tissue is homogeneous.

c) Different enzymes respond to protein depletion in different ways. In most cases activity is decreased, but sometimes it is unchanged or even increased (8, 9). There seems to be little rhyme or reason about these responses. It is possible to make teleological hypotheses in terms of 'adaptation,' 'disuse atrophy,' or preservation of the more essential enzymes at the expense of the less essential. At present, however, it would be difficult to predict with any confidence the changes produced by a given nutritional stress. One aspect that has not been systematically studied is to compare the behaviour of soluble and particulate enzymes.

d) Different organs differ in their response. Very often enzymes seem to be reduced in the liver when they are well-maintained in other organs, such as kidney and myocardium (8, 10, 11). Perhaps this is because the liver contains a large proportion of so-called 'labile' protein, which is stored in the form of enzymes.

e) Even when a definite change in enzyme activity has been produced, it may not have the physiological significance that might be expected. For example, rats in whom liver and kidney xanthine oxidase activity, as measured *in vitro*, has been reduced to very low levels by protein deficiency, are still able to convert xanthine to allantoin *in vivo* (12, 13). The same has been found with enzymes for which pyridoxine and biotin act as coenzymes. These systems are involved in the metabolism of nitrogen compounds —transamination and urea formation. *In vivo*, in contrast to findings *in vitro*, even severe deficiency caused only mild disturbances in N metabolism (14, 15).

RESULTS IN MAN

It is only in recent years that measurement of enzyme activity in blood, digestive secretions and tissues has been developed as a tool for the investigation of human malnutrition. This tool has mainly been used to study the deficiency disease known in the English-speaking world as kwashiorkor (3). The approach is logical, because although kwashiorkor has many names and many variants in different countries, there is general agreement that its principal cause is a deficiency of protein of good biological value. The disease develops in infants who, after weaning, are put onto a diet consisting mainly of carbohydrate. The nature of the carbohydrate source varies from region to region—in Europe it is refined wheat flour; in Mexico and Central America corn; in Central Africa cassava; in the West Indies cornmeal and sugar. The calorie supply is therefore fairly good —at least until anorexia sets in—and it is this that differentiates kwashiorkor from starvation or marasmus. The distinction, although useful in theory, is often obscured in practice. Both calorie and protein intakes may vary over a wide range, so that intermediate and mixed forms of malnutrition occur, which present some of the features of kwashiorkor, but not the fully developed picture. The term 'protein malnutrition' has been introduced to indicate the underlying process that is a common factor in these varied clinical states (16).

Although kwashiorkor has been described in adults (3), it is predominantly a disease of infancy and early childhood. In adults the effects of protein deficiency appear to be less dramatic and less common, except in times of emergency, such as famine and war, when biochemical investigations often cannot be done. An exception is the comprehensive study of McCance and his coworkers in Germany (17). In general, however, the information available about enzyme changes in malnourished adults is scanty. Almost all the work described below was done on babies and children.

Studies on enzymes in protein malnutrition have had 3 objectives: *1*) to help in the diagnosis of a specific disease process—i.e. dietary protein deficiency; *2*) to assess the degree of deficiency and the response to treatment; *3*) to provide evidence of functional damage to organs such as liver and pancreas. The results that have been obtained, and the extent to which these objectives have been realized, will be considered under 3 headings: enzymes in blood, in digestive secretions, and in tissues.

Enzymes in Blood

Much of the work that is relevant to our problem has been concerned with the ability of serum or plasma to split ester linkages in substrates of various kinds. It is still an open question how far the esterases concerned are specific, or whether some at least are able to act on a wide range of substrates (18). The problem cannot be dismissed as academic. It is important to distinguish specific

enzymes wherever possible, because they may have their origin in different tissues.

Cholinesterase. McCance and his colleagues, working in Germany after the second world war, made a systematic study of the cholinesterases of serum and red cells in malnourished adults (19, 20). The enzyme in red cells is mainly the specific or 'true' cholinesterase (T-enzyme), according to the classification of Mendel, Mundell and Rudney (21); in serum the non-specific 'pseudo'-cholinesterase (P-enzyme) predominates, but there is also a small amount of the T-enzyme. Malnutrition did not affect the cholinesterase activity of the red cells, but the activity in serum was much reduced. The P-enzyme was affected more than the T-enzyme. The reduction was greater in patients with famine edema than in those without edema, and seemed to be roughly proportioned to the degree of dietary deficiency.

All subsequent work on cholinesterase in malnutrition has been concerned with the 'pseudo' enzyme of serum. Waterlow (22) found low levels in malnourished infants in West Africa. This has been confirmed by Dean and Schwartz (28) in Uganda, by Jelliffe, Bras and Stuart (24) in Jamaica, and by Scrimshaw and his colleagues in Guatemala (25, 26).

The reduction in serum cholinesterase is not confined to cases presenting the clinical picture of classical kawashiorkor. It is found also in intermediate cases, of the type called 'marasmic kwashiorkor' by Jelliffe *et al.* (24), and in many babies with general undernutrition or marasmus. No comparative measurements have yet been published of the cholinesterase levels in these different clinical forms of malnutrition. In a series of cases in Jamaica the following results were obtained (27):

	No. of Cases	Serum Cholinesterase Activity, Units*	Range
Kwashiorkor	23	0.299±0.019	0.15–0.65
Marasmic kwashiorkor	20	0.296±0.033	0.08–0.68
Marasmus	13	0.322±0.047	0.11–0.70
Normal level		0.6–0.8	

* Mean ± S. E.

There is no significant difference between the means in the 3 clinical groups. It is therefore clear that a reduction in serum cholinesterase activity is not correlated with a specific clinical picture, and is in no sense diagnostic of kwashiorkor. It has been recognized for a number of years that the enzyme is reduced in debilitating conditions where there is secondary malnutrition, such as tuberculosis or cancer (28). This could be the result of a common underlying process—protein-malnutrition or protein depletion.

In our experience, measurement of serum cholinesterase activity has been of practical value, in spite of the wide range of variation shown in the table above. When measurements are made by the method of Michel or one of its modifications (29), the normal level is about 0.6–0.8 units. In severely malnourished babies the activity may fall to 0.15 units or less. A reduction of this degree is greater than that found in serum albumin concentration, which seldoms falls below about 1 gm/100 ml—i.e. ¼ of normal. Cholinesterase, like albumin, rises rapidly with successful treatment (22, 23). If this rise does not occur, the prognosis is bad.

Nothing is known for certain about the physiological function of the pseudo-cholinesterase of serum. In animals it is present in some species but not in others. In the rat the activity is much higher in the serum of females than of males (30). In man the enzyme is apparently not essential for life, since when pseudo-cholinesterase activity was inhibited by difluorophosphate, no harmful effects were observed (31, 32). To this extent, therefore, measurement of the enzyme as an index of protein malnutrition is empirical.

The test, nevertheless, has some rational basis. Animal experiments indicate that the pseudo-cholinesterase of serum is formed in the liver (33). Many years ago it was shown that the serum activity is reduced in liver disease (34, 35). In our work in West Africa we found that the cholinesterase activity of liver tissue (obtained by needle biopsy) was low when the serum activity was low, and vice versa (36). Perhaps, therefore, it is reasonable to regard the serum cholinesterase level as an index of the ability of the liver to form new protein. This may be impaired either by local disease, as in cirrhosis, or by a shortage of raw materials, or by infection, as happens with the bone-marrow.

Other Esterases. In malnutrition the ability of the serum to split other esters seems to be reduced to about the same extent as the pseudo-cholinesterase activity. It may well be that although the substrates are different, the enzyme is the same.

Srinivasan and Patwardhan (37) in India

measured the esterase activity of plasma, using ethyl butyrate as substrate. In children with kwashiorkor the average activity on admission to hospital was 67 units, rising to 154 units after treatment. This final level was identical with that found in a group of normal controls. The Indian workers regard the plasma esterase activity as a measure of liver function. They found a rough correlation between the esterase level in plasma and the degree of fatty infiltration of the liver, as judged microscopically. Similar results were obtained by Dean and Schwartz (23) with phenyl benzoate as substrate. They found that in kwashiorkor the plasma esterase activity was about ⅓ of normal, rising rapidly with treatment.

It has long been known that human plasma contains an enzyme capable of hydrolysing lower triglycerides. In 1912 Bauer (38) observed that the tributyrin-splitting activity of plasma was reduced in cachectic subjects. Srinivasan and Patwardhan (37) claim that plasma contains a true lipase, which they believe to be derived from the pancreas. They measured the activity of this lipase, with tributyrin as substrate, in a series of infants with kwashiorkor; the mean activity was only ¼ of that found in normal controls. They suggest that this test may be a more convenient method of determining pancreatic function than duodenal intubation.

Mukherjee and Werner (39), also working in India, found no reduction in plasma lipase activity in malnourished adults. They used olive oil as a substrate, and it is possible that this difference in technique, as well as in the age of the patients, may account for the discrepancy. As will be seen below, there is convincing direct evidence of failure of pancreatic function in malnourished infants, but as far as we are aware no studies of the same kind have been made in adults.

In some cases indirect evidence about enzyme activity may be obtained by measurement of substances in blood that are not themselves enzymes. Dean and Schwartz (40) suggested that the low serum levels of total and esterified cholesterol which they and others (41, 42) have found in kwashiorkor might result from decreased activity of the enzyme cholesterol esterase.

Amylase. Amylase is another enzyme whose presence in the blood probably represents a 'spill-over' from the pancreas. There is a measure of agreement that in kwashiorkor serum amylase levels tend to be low. This has been observed by Dean and Schwartz (23) in Uganda, by Behar and his colleagues (25) in Guatemala, and by De Maeyer and Vanderborght (41) in the Belgian Congo. On the other hand Dricot, Beheyt and Charles (43) found that in half their cases the amylase level was slightly raised. The Guatemalan workers regard the serum amylase level as a useful prognostic index in the individual patient. This view is not shared by De Maeyer and Vanderborght (41). They found a wide range of variation in normal subjects; in many undernourished children the serum amylase level fell within this range, so that in their view only group means are significant.

In adults suffering from undernutrition Perakis and Bakalos (44) recorded high values for serum amylase (diastase). On the other hand Mukherjee and Werner (39), also working on adults, found that serum amylase activity was reduced whenever the serum albumin concentration had fallen below 2.5 gm per 100 ml. Below this level there was a linear correlation between the serum concentration of albumin and of amylase. Glaser (45) measured the urinary amylase output in 10 undernourished men in Germany. In 9 it was normal, and in one low.

Alkaline Phosphatase. Divergent results have been reported for the alkaline phosphatase activity of the serum in kwashiorkor. Waterlow in Jamaica (2) found normal levels both in cases of 'fatty liver disease' (now considered to be the same as kwashiorkor) and in a control series of marasmic babies. Normal levels were also found in undernourished infants in Ceylon (46). Dricot *et al.* (43) found the activity low in 2 cases and high in 2 others.

More recent studies have agreed in showing that low alkaline phosphatase levels seem to be characteristic of kwashiorkor. Schwartz (47) found an average level of 14 units[1] in severe cases, rising to 35 units after 6–10 wk. treatment. Reductions of the same order were recorded by the group working in Guatemala (25, 26). In a later series of cases in Jamaica the average initial activity was 10 units;[2] after recovery it was 2[illegible] units (48). The reason for the discrepancy with the earlier work is not clear. It is of interest that

[1] Schwartz's unit is the amount of enzyme liberating 1 mg. P from sodium-glycerophosphate in 3 hr. at 37°. This unit is slightly greater than the King-Armstrong unit.

[2] The unit used in Jamaica is the King-Armstrong unit. (For these results we are grateful to Dr. L. N. Went.)

in Brazil Budiansky (49) observed that cases of rickets did not show the high levels of alkaline phosphatase characteristic of that disease in Europe and North America.

Both in Uganda and in Jamaica the alkaline phosphatase levels seem to vary over a rather wide range. In the Jamaican series the variability is greater than for cholinesterase. This suggests that the alkaline phosphatase activity may be determined by more than one factor, and fits in with the hypothesis of Dean and Schwartz (23) that the activity as measured may represent the sum of different enzymes from different sources. Schwartz (47) in a detailed study, has shown that during treatment there is a change in the pattern of activation by magnesium and of inhibition by cyanide. She says "it is difficult to explain this course of events without postulating the presence of at least three alkaline phosphatases in the serum." So far the origin of these phosphatases has not been identified, but the direction of this work is an excellent example of the way in which the study of enzymes in the blood may provide evidence of functional changes in different organs.

Transaminase. Wroblewsky and Ladue (50) have shown that in liver disease serum transaminases are increased. In malnourished babies the glutamic-oxalacetic transaminase activity was in many cases higher than in normal or treated infants (51). In both groups, however, the range of variation was rather large. This variability is also apparent in the figures given by Cove *et al.* (52) for serum transaminase in newborn infants. The fact that a rise has been found in malnutrition suggests that there may be an element of tissue destruction, in the liver or elsewhere. So far no correlation has been observed with the severity of the general condition.

Other Enzymes. Hutchinson (53) studied the activities of carbonic anhydrase, catalase, acid phosphatase, glyoxalase and 'true' cholinesterase in the red cells of men with hunger edema. There was some evidence of a slight increase in carbonic anhydrase activity in the most undernourished subjects, but no changes were found comparable to those that occur in the serum. De Marco and Cusmano, (54) on the other hand, found low carbonic anhydrase activity in the blood of dystrophic infants.

Enzymes in Digestive Secretions

Saliva. It has long been known that in malnutrition the parotid glands may become enlarged, particularly in adults. The literature has been reviewed by McCance, Dean and Barrett (55). The histological basis of this enlargement appears to be a hypertrophy of the secretory acini, with increased granulation of the cells (57). In infants with kwashiorkor, on the other hand, the salivary glands are often atrophic, with degranulation of the zymogen-secreting cells, and in the later stages cellular infiltration and fibrosis (58, 59). It seems likely therefore that in malnutrition salivary function is affected, but as far as we are aware no work has been done on the volume or enzyme activity of saliva.

Gastric Juice. There is evidence that in kwashiorkor gastric secretion of acid is depressed. This might result from deficiency of the enzyme carbonic anhydrase. Gillman and Gillman (57) say that in their patients free acid was not detected in a single instance. On the other hand, in Jamaica Waterlow (6) found that infants with fatty liver and edema had a lower pH in the resting gastric juice, and tended to produce more acid after a test stimulus (brandy) than babies with pure marasmus, who did not have fatty livers. The pepsin content of the gastric juice was not measured. Jayakasera *et al.* (46) found that in malnourished infants in Ceylon the total acid produced in response to histamine was only about half the amount expected in European children. All their cases showed some peptic activity.

Pancreatic Secretion. In 1925 Jackson (60) described the atrophy and degranulation of the pancreatic acinar cells that result from inanition. These pathological changes in the pancreas are very striking in infants dying of kwashiorkor, and have been recorded in several parts of the world (61–63). Less commonly, and perhaps in the more chronic cases, the atrophy is accompanied by other changes—fatty infiltration, dilation of ducts with retention of colloid, and even diffuse fibrosis (66, 64, 65).

These pathological lesions are accompanied by functional changes. Veghelyi (66, 67) studied 109 children during the siege of Budapest in World War II, when milk became unavailable. Within about 3 weeks there was a fall in the enzyme activity of the duodenal juice. First lipase and trypsin fell, and then amylase. These changes began before malnutrition was severe, before edema occurred or there was any evidence of liver damage. They could not be prevented by vegetable foods or vitamins, and responded only

to milk. Similarly in Egypt Badr El-Din and Aboul Wafa (68) found that in malnourished babies trypsin began to diminish at an early stage, before the development of edema or liver enlargement.

Observations of the same kind have been made in kwashiorkor. Magalhaes Carvalho, Schmidt and Pinto (69) found low levels of trypsin and lipase in convalescent children in Brazil. Dricot *et al.* (43) in the Belgian Congo recorded reductions in lipase, but in 3 out of their 4 cases trypsin activity was normal. Two very detailed studies of this problem have been made, by Thompson and Trowell (70) in Uganda, and by Gomez and his colleagues in Mexico (71). The agreement between these 2 groups of workers is striking. The enzyme levels on admission were $\frac{1}{3}$–$\frac{1}{10}$ those found at the end of treatment. In Uganda the greatest reduction was in trypsin, in Mexico it was greatest in lipase. Gomez and his colleagues made some experiments in which digestion and absorption were tested by measuring blood amino-acid levels after a dose of gelatin or calcium caseinate. Slight abnormalities were found, suggesting a deficiency in both absorption (or digestion) and utilization. In the Uganda cases serial measurements of duodenal enzyme activity were made in the course of treatment, and showed that pancreatic function may be restored to normal very rapidly. Trowell, Davies and Dean (3) reproduced a most striking chart, showing the parallel rise during treatment of duodenal and blood enzymes (amylase and esterase).

The evidence, therefore, is strong that in kwashiorkor there is a failure of pancreatic enzyme production. Quite different results, however, have been obtained by several workers in England and North America who have measured duodenal enzyme activity in malnourished infants. McDougall (72) found normal activity in 18 malnourished babies, mostly less than 6 months old. Matsaniotis (73) measured the plasma amino- N curve after feeding casein or casein hydrolysate. He found no significant differences in malnourished infants. The mean age of his cases was 15½ months, which is about the age when kwashiorkor develops; they were about 50% below normal weight, and so in severity corresponded to '3rd degree malnutrition' of Gomez *et al.* The results therefore cannot be explained by the factors of age or severity. Bate and James (74) made a similar study in 8 babies who were about 30% below weight. The amino-acid curves were somewhat low, but in 3 out of 4 cases tested duodenal trypsin content was normal.

A possible explanation of this discrepancy is that kwashiorkor and marasmus in their extreme forms are different diseases, and that pancreatic failure is a specific feature of one disease process—kwashiorkor—and not of the other. This point of view has been put forward by Trowell (16). The only comparative study that seems to have been done of duodenal enzymes in the 2 types of case is that of Badr El-Din and Aboul Wafa (68). They found that trypsin was reduced both in kwashiorkor and in marasmus. The opinion of pathologists originally inclined to the view that pancreatic damage was specific to kwashiorkor. Davies (3) wrote "in marasmus, although the cells of the pancreatic acini may be slightly atrophic, they contain secretory granules in abundance." Waterlow (2) found a normal appearance of the acini in some infants dying of marasmus. However, more detailed studies by Bras and his colleagues in Jamaica (64, 65) have shown that the position is not so simple. Many marasmic babies at autopsy did show pancreatic changes, which were much less common in a control series of children dying from non-nutritional diseases. Of the babies diagnosed as marasmus many had fatty livers, and there was a statistically significant association between fatty liver and pancreatic lesions. Equally, marasmic infants with normal livers tended to have normal pancreases. These findings recall the suggestion made by Davies (62), that perhaps the pancreatic atrophy causes the fatty liver, and the observation of Veghelyi (66) that pancreatic secretory failure precedes evidence of liver damage.

In animal experiments an explanation put forward for the lipotropic activity of pancreatic juice and pancreatic extracts is that by their proteolytic action they increase the availability of methionine (75). Whether this explanation holds for human beings we do not know. In general in kwashiorkor there seems to be surprising little impairment of the digestion and absorption of food, at least with diets based on milk (3, 76, 77).

Not enough work has been done on pancreatic function in malnutrition to determine how far reduction in enzyme activity correlates with the severity of the clinical condition. Gomez *et al.* (71) could find no relation between duodenal enzyme content and severity, as judged by the

plasma albumin level. This problem could be tackled more easily, once it has been shown that serum lipase and amylase levels do in fact reflect the quantity of these enzymes in duodenal juice.

The Succus Entericus. The mucosa of the small intestine shares in the atrophy undergone by other epithelia. Davies (3) says: "exocrine enzyme secreting cells undergo the same profound atrophy as the pancreatic acinar cells, with reduction in size and lack of granularity." Perhaps this is the cause of the noninfective diarrhea that so often accompanies undernutrition, in both adults and infants (3, 78), with the passage of undigested food in the stools. It is probable that enzyme secretion is reduced, but in human malnutrition, as far as we are aware no direct measurements have been made of enzymes in intestinal contents below the duodenum. In protein-deficient rats a reduction has been found of the enzyme prolidase (79).

Enzymes in Tissues

Studies of tissue enzymes in human malnutrition have been concentrated on the liver, and have been made possible by the technique of needle biopsy. It is surprising that so little attention has been paid to muscle, considering its accessibility. Perhaps the reason for this is that much less experimental work has been done on this tissue.

In the small pieces of tissue obtained by needle biopsy there is the possibility of a large sampling error, and it is therefore desirable to have some idea of the reproducibility of the results. In studies in West Africa satisfactory agreement was found between measurements on 2 parts of the same biopsy core (36). Although the samples must have come from closely adjacent parts of the liver, they were large enough to contain at least fragments of several lobules. These measurements were made on infants with malnutrition, in whom the pathological process in the liver, if any, is diffuse and uniform (80). In livers with focal necrosis or cirrhosis it is much less likely that a single biopsy would be representative (81).

A second technical difficulty has already been alluded to—that of the basis of reference. In biopsy specimens the best basis in theory is probably the DNA content. In practice, however, this is not ideal. There is a rather large variability between duplicate micro-samples from the same liver (82). This may be because of the lymphocytic infiltrations that are so common in the malnourished liver (57).

The first enzyme to be studied in the human liver in malnutrition was cholinesterase (22). In our work in West Africa (36), it was found that the activity in liver varied in the same way as in plasma, being low on admission to hospital and rising on recovery. Mukherjee (83) found an even more striking reduction in cholinesterase in the livers of adult patients with nutritional edema, as well as in cases of kwashiorkor. Later in our laboratory the study was extended to a wider range of enzymes catalysing single-step reactions. The subjects were malnourished infants, some but not all of whom had fatty livers. In all cases there was evidence, both clinical and from the dietary history, of severe protein depletion. The enzymes measured were lactic, malic and glutamic dehydrogenases, glutamic-oxalacetic transaminase, cytochrome-c DPN reductase, and cytochrome oxidase (84, 85). Comparisons were made in the same case before and after treatment. None of these enzymes were found to be reduced in the malnourished liver, and indeed there was evidence that 2 enzymes—transaminase and malic dehydrogenase—had a higher activity per unit weight of tissue in malnutrition than after recovery. It was interesting that there was no change in the activity of cytochrome-c reductase, in spite of the fact that this is a flavoprotein enzyme, and that some of the patients had clinical signs of riboflavin deficiency. In the course of this work it was noted that the pattern of enzymes in the human liver was quite different from that in the rat—the activity of all the oxidative enzymes is much lower, by a factor of about 3. That is to be expected in a larger animal, but it was unexpected to find that transaminase activity was much higher in the human liver.

This work was confirmed and extended by Scrimshaw and his colleagues, in a study of 13 children with kwashiorkor (86). They found also that transaminase and malic dehydrogenase activity (expressed per unit weight of protein) were slightly higher in the initial than in the final biopsy. These workers made a special study of riboflavin and the flavin-containing enzymes, of which they selected 4 examples, glycolic acid oxidase, DPNH-dehydrogenase, xanthine oxidase and d-amino-acid oxidase. The first two of these were equally active in livers from malnourished and recovered children, nor was there any difference in total riboflavin content. On the other hand, in the malnourished liver there was a striking and consistent fall in xanthine oxidase

and d-amino-acid oxidase activity, to about ⅓ of the level found after recovery. These results agree well with those that have been obtained experimentally in protein-deficient rats (87–89).

Mukherjee in Calcutta, in a study of 12 malnourished adults and 7 children, found that in both groups catalase levels were about twice as high (expressed per unit nitrogen) in the initial as in the final biopsy (83). The iron content was also more than twice as great. It has been suggested that the increased iron content of the liver in malnutrition, which may be demonstrated histologically as well as chemically (2), could result from failure of utilization because of lack of protein for building hemoglobin. Perhaps catalase, like cytochrome reductase, is maintained because of its great importance in cellular metabolism.

Mukherjee (83) also showed that alkaline phosphatase activity was much higher in the malnourished liver than after recovery. This confirms the results obtained histochemically by Sriramachari and Ramalingaswami (90). Schwartz (47) has discussed the apparent paradox that liver phosphatase should be high when serum phosphatase is low—a picture that has also been found in animal experiments (91). She suggests that the liver may normally inactivate alkaline phosphatase, and that this disequilibrium arises from a simultaneous depression of enzyme formation and destruction.

In any investigation of tissue enzymes in protein deficiency it is natural that attention should be paid as far as possible to enzymes concerned with nitrogen metabolism. It is for this reason that transaminase has been studied by the groups in Guatemala and in Jamaica. Sposito and Russo-Caia (92) in Rome measured dipeptidase activity in biopsy specimens from normal and pathological livers. Their results show a rather wide range of scatter, but in fatty livers the activity seemed to be a little low. It is conceivable also that cholinesterase might be concerned with protein metabolism, since it has been shown that the 'true' enzyme can mediate N- as well as O-acylation (93). Whether this is the real function of the enzyme is not known.

In a way the results of these tissue studies may seem disappointing. They do not help in the diagnosis of protein-depletion or the assessment of its severity, and they give little insight into the nature of the biochemical derangement, if any. On a teleological interpretation an enzyme that is lost is perhaps an enzyme of little importance. Again, it is probably naive to expect large changes in the activity of any essential enzyme, since quite small changes could, by their cumulative effects, cause profound alterations in metabolic pattern. Moreover, it is logical to suppose that the most sensitive systems may be those that are most highly organized, whose activity depends in some degree upon structural integrity. Misinterpretations may arise if this factor is neglected. A possible example is the high levels that have been found for transaminase and malic dehydrogenase activity in the malnourished liver. Both these enzymes are partly intramitochondrial. As Judah and Christie have shown (94), when measurements are made in sucrose homogenates, a high level of activity may merely indicate mitochondrial damage and increased permeability.

As a first attempt on the problem of detecting changes in the organization rather than in the amount of enzymes, measurements have been made of the oxidation of a Krebs cycle substrate (malate); of oxidative phosphorylation; and of phosphate uptake into phosphatides (95). These are systems of increasing complexity, in that the second depends on the first, and the third on the second. In malnourished liver there was no reduction in malate oxidation, but a significant fall in phosphorylation, and a variable reduction in the uptake of phosphate into phosphatides. In other words, there was some degree of uncoupling of oxidation and phosphorylation.

If this effect occurred *in vivo*, the results could clearly be very serious. It has been suggested, for example, that the potassium depletion and intracellular accumulation of sodium that are found in severe chronic malnutrition might result from failure of the energy-producing mechanism on which depends the integrity of the intracellular electrolyte pattern (96).

DISCUSSION

It has been known for nearly 100 years that when an animal or human being is changed from a high to a low protein diet, there is a transitional period of negative nitrogen balance. Allison (97) has shown very clearly that an animal may be in nitrogen equilibrium at many different levels of intake. In moving from one level to another it gains or loses protein. This protein, which is easily mobilized and easily built up again, has been thought of as a kind of reserve. An animal on

a low protein intake, although in nitrogen balance, may be described as having depleted protein stores. This is an important concept, but on verbal grounds it is open to misunderstanding. No one has succeeded in demonstrating the existence of a storage protein, in the sense of a material specifically available for the replacement of other proteins, without any structural or functional role of its own. It seems, therefore, that when protein is lost from the body as a result of a low protein intake, it must be derived from the cytoplasm of functioning cells, and not from any specific site or depot.

All organs and cells do not lose protein to the same degree. Addis, Poo and Lew (98) showed that in the fasted rat the liver lost some 40% of its protein in the first 4 days, whereas other organs, such as brain and heart-muscle, were very little affected. This has been confirmed by many subsequent workers, both in animals and in man (99–101).

Since enzymes are complex proteins, it is not surprising that they share in this loss. Therefore nitrogen equilibrium can hardly be considered a satisfactory criterion of an adequate nitrogen intake, if a subject, although in balance, has a depleted battery of tissue enzymes. On the other hand, it must be admitted that we cannot yet say for certain whether the loss of enzymes is in fact a disadvantage. Allison (102) has found that the dog with reduced liver enzymes is more vulnerable to certain kinds of poisons. It is a problem for the future whether these findings can be extended to all forms of stress.

As was mentioned in the introduction, a protein-deficient diet affects different enzymes in different ways, even in one organ such as the liver. Some are rapidly lost, while others are selectively preserved or may even be relatively enriched. No pattern has yet emerged which explains why this should be so—whether it is a homoeostatic mechanism, or whether the preservation of one enzyme and the loss of another results in some way from the particular nature of the diet. On general grounds it is to be expected that not only enzymes but also other cell proteins will react in a non-uniform way. This is because different proteins are synthesized and broken down at different rates. In recent years those who study protein metabolism by isotopic techniques have come to realize that to speak of the half-life or turnover time of 'liver protein' or 'muscle protein' is inexact. Organs contain a spectrum of proteins with different turnover rates, and what is in fact measured is an average. Presumably, although there is no evidence of this, enzymes also differ among themselves in the same way.

From the beginning, therefore, protein depletion probably causes a non-uniform loss of enzymes. But this pattern cannot be static. As Addis *et al.* showed (103), with continuing protein deficiency the loss from internal organs such as liver and pancreas is halted and the main burden is taken up by the muscles. How this redistribution is brought about is unknown, but the fact is important. It means that the pattern of effects produced by protein depletion must constantly be changing. If this is true, it is unlikely that measurement of a single enzyme in one tissue could ever be of much value for diagnosis or assessment. From the clinical point of view, if the pattern is changing as deficiency is prolonged, it follows that the factor of chronicity must always be considered. Many of the differences that have been described in kwashiorkor in different parts of the world may be caused not so much by the nature of the diet both in its quantitative and qualitative aspects, as by variations in the speed and duration of depletion.

One of the reasons why internal organs such as liver and pancreas are the first to lose cytoplasmic protein when the intake is low, probably lies in the fact that they have a high protein turnover. Their synthetic activity must therefore be closely dependent on an intact supply-line. In the pancreas an early effect of a protein-deficient diet is a reduction in the secretion of trypsin and amylase (70, 71). For the clinician it is natural to think of this in terms of its effect upon digestion. From the metabolic point of view it may be more useful to regard these enzymes as examples of protein produced by a tissue with a very high rate of turnover. In this sense it is perhaps a matter of detail that one type of protein is discharged into the digestive tract, another, such as plasma albumin, into the blood stream, whereas a third may never leave the cell in which it is formed. The fact that one protein travels more widely than another does not necessarily imply an essential difference in metabolic behaviour.

The reason for making this point is that in human subjects a good deal of work has been done on enzymes in the bloodstream and in digestive juices. These results have sometimes been regarded as empirical, since the physiological importance of enzymes in the blood is in most

cases unknown, and even digestive enzymes seem to be to some extent dispensable. In kwashiorkor, for instance, in spite of the reduction in pancreatic enzymes there is very little evidence of malabsorption (77). It may be more fruitful to look upon the enzymatic activity of proteins in blood and external secretions as a kind of label. By studying the proteins labelled in this way we may get information about the activity of the organs or tissues from which they are derived (104, 105). These enzymes are therefore useful tools for distinguishing different patterns of protein metabolism that may result from depletion of varying duration or severity.

Up to this point the argument has been a general one—that enzymes, being proteins, share in the depletion produced by a deficient intake. That the effect occurs is well-established, but of its mechanism we know almost nothing, and in the present state of knowledge to speculate about details is not very profitable. It may, however, be useful to distinguish broadly between 2 types of mechanisms. The first is what might be called depletion by exhaustion. A failure in the total supply of protein must in the end lead to an over-all failure of protein synthesis. Before the end is reached, while protein synthesis is gradually running down, for the reasons given above there will not be a uniform loss of all proteins and enzymes, but a highly complicated pattern of selective loss, depending upon turnover rates and interchanges between one organ and another. But the fact that there is a complicated pattern does not rule out a very simple single cause—a general failure of supply.

In certain situations, however, more specific causes may operate. It has been suggested, for example, that deficiency of sulphur amino-acids might lead to a selective loss of sulphur-containing enzymes (3). No convincing evidence of such an effect has been produced. Although many important enzymes depend for their activity on sulphydryl groups, this does not mean that they have a greater content of sulphur amino-acids. In fact, there is no reason to suppose that cellular proteins, enzymatic or otherwise, differ much in their amino-acid content. Modern work on the specificity of proteins emphasizes differences in amino-acid sequence and in the configuration of peptide chains rather than in composition. This does not apply, of course, to extracellular proteins such as collagen, which contains almost no sulphur, and keratin, which contains an excess (106).

Another mechanism that might operate in some cases is that of substrate adaptation. This phenomenon is very well-known in bacteria. Its occurrence in higher animals has been reviewed by Knox, Auerbach and Lin (7). An example is the dependence of the arginase activity of the liver on the protein content of the diet. The concept is important, since it emphasizes the fact that differences in the proportions of carbohydrate, fat and protein in the diet may lead to differences in the enzyme pattern, and hence of metabolic pathways. Enzyme depletion produced in this way could be called loss from inactivity rather than from exhaustion.

Many of the dietary conditions that have been used experimentally to produce adaptations or changes in enzyme activity are highly artificial creations of the laboratory, and are unlikely to be reproduced in human diets. It is doubtful if deficiency of a single amino-acid could occur in practice. If one or more essential amino-acids are limiting, the nonessentials present in excess are excreted, so that the over-all effect is that of a low total protein intake. The word deficiency, however, does not cover all the ways in which a diet may be unsatisfactory. Increasing attention is being paid, both in animal experiments and in man, to the possible harmful effects of a diet in which the total protein intake is adequate or even high, but the amino-acid pattern unbalanced (6). This tends to occur, for instance, in diets based on maize. It may be supposed that if amino-acid imbalance does produce harmful effects different from those of total protein deficiency, it must be associated with specific enzyme changes. This is a subject for future research; at present no evidence is available.

Examples have been given in the introduction of situations in which an enzyme, as measured *in vitro*, seems to be almost completely lost (e.g. xanthine oxidase, transaminase) yet there is little or no effect on over-all metabolism. One must conclude that the measurement *in vitro* is artificial. Very often this results from the very nature of the measurement. The conditions of assay are so chosen that there are no limiting factors other than the amount of enzyme. In the cell, however, there are many limiting factors, such as substrates, cofactors, electrolytes and trace metals, and no single enzyme acts independently of others. Although enzymes undoubtedly exist as chemical entities, from the physiological point of view a single enzyme is an abstraction. Of more physiological significance,

therefore, may be measurements involving a chain of enzymes, such as glycolysis, oxidative phosphorylation, or synthetic reactions. Most of these complex multi-enzyme systems depend upon some degree of intact structure. The structural units are not yet precisely defined, since the recent work of Green and his school and of Lehninger has shown that by suitable methods particles can be prepared at will, capable of catalyzing varying numbers of steps in a complex sequence (107, 108). But for all reactions of this type, and particularly for synthetic reactions coupled to energy production, there is *some* structural integrity that has to be preserved. The possibility must therefore be considered that when a low-protein diet is found to cause a change in the activity of some reaction, this may be due to a change in the structural relationship of enzymes, rather than in their amounts.

CONCLUSION

In this discussion we have considered in general terms the nature of the relation between enzyme activity and dietary protein intake. For practical purposes we can reach certain conclusions: *1*) It is unlikely that measurement of any one enzyme or system in one tissue could be used as a general index of protein malnutrition, because, as deficiency progresses, the pattern of enzymatic activity in different organs must constantly be changing. *2*) However, measurement of appropriate enzymes in blood and digestive juices probably does give information about the protein synthetic or secretory activity of individual organs. *3*) It is possible that specific types of amino-acid deficiency or imbalance in the diet might lead to characteristic tissue enzyme changes which would be diagnostic. So far, however, no example of such a relationship has been established. *4*) Even when there is a definite effect of diet on enzyme activity, the significance of this for the body as a whole is often obscure.

Thus, from the strictly practical point of view—the use of enzyme measurements for the diagnosis and assessment of protein malnutrition—the picture does not seem very encouraging. But the work that has been done in the last 10 years is stimulating new concepts, and suggests many points for a new experimental approach.

I wish to thank Professor Paul György for his encouragement and interest, and for his great help in summarizing the experimental literature.

REFERENCES

1. Munro, H. N. *Physiol. Rev.* 31: 449, 1951.
2. Waterlow, J. C. *Spec. Rep. Ser. med. Res. Counc.* (No. 263) London: Her Majesty's Stat. Off., 1948.
3. Trowell, H. C., J. N. P. Davies and R. F. A. Dean. *Kwashiorkor.* London: Arnold, 1954, p. 59.
4. Waterlow, J. C., G. Bras and E. Depass. *J. Trop. Pediat.* 4: 189, 1957.
5. *Proceedings of a Conference in Jamaica,* edited by J. C. Waterlow. Rome, F.A.O., 1955, p. 2.
6. *Proceedings of a Conference in Princeton,* edited by J. C. Waterlow and J. M. L. Stephen. Rome: F.A.O., 1957, p. 32.
7. Knox, W. E., V. H. Auerbach and E. C. C. Lin. *Physiol. Rev.* 36: 164, 1956.
8. Wainio, W. W., B. Eichel, H. J. Eichel, P. Person, F. L. Estes and J. B. Allison. *J. Nutrition* 49: 465, 1953.
9. Rosenthal, O., J. C. Fahl and H. M. Vars. *J. Biol. Chem.* 194: 299, 1952.
10. Wainio, W. W., J. B. Allison, B. Eichel, P. Person and G. R. Rowley. *J. Nutrition* 52: 565, 1954.
11. Awapara, J. *J. Biol. Chem.,* 200: 537, 1953.
12. Williams, J. N., Jr., P. Feigelson and C. A. Elvehjem. *J. Biol. Chem.* 185: 887, 1950.
13. Bass, A. D., J. Tepperman, D. A. Richert and W. W. Westerfield. *Proc. Soc. Exper. Biol. & Med.* 73: 687, 1950.
14. Terroine, T. *Arch. sc. physiol.* 4: 25, 1952.
15. Terroine, T. and P. Rombauts. *Arch. sc. physiol.* 6: 25, 1952.
16. *Joint FAO/WHO Expert Committee on Nutrition.* (Report of 3rd Session). WHO Technical Report Series No. 72, Geneva, 1953; FAO Nutrition Meetings, Report No. 7, Rome, 1953.
17. McCance, R. A. *Spec. Rep. Ser. med. Res. Counc.* (No. 275) London: Her Majesty's Stat. Off., 1951.
18. Ammon, R. and M. Jaarma. In: *The Enzymes,* edited by J. B. Sumner and K. Myrbäck. New York: Acad. Press, 1950, vol. 1, pt. 1, p. 390.
19. Hutchinson, A. O., R. A. McCance and E. M. Widdowson. *Spec. Rep. Ser. med Res. Counc.* (No. 275) London: Her Majesty's Stat. Off., 1951, p. 216.
20. McCance, R. A., E. M. Widdowson and A. O. Hutchinson. *Nature, London* 161: 56, 1948.
21. Mendel, B., D. B. Mundell and H. Rudney. *Biochem. J.* 37: 473, 1943.
22. Waterlow, J. C. *Lancet* 1: 908, 1950.
23. Dean, R. F. A, and R. Schwartz. *Brit. J. Nutrition* 7: 131, 1953.
24. Jelliffe, D. B., G. Bras and K. L. Stuart. *West Indian Med. J.* 3: 43, 1954.
25. Behar, M., G. Arroyave, C. Tejada, S. Viteri and N. S. Scrimshaw. *Rev. col. med. Guatemala* 7: 221, 1956.
26. Scrimshaw, N. S., M. Behar, C. Perez and F. Viteri. *J. Pediat.* 16: 378, 1955.

27. Stuart, K. L., G. Bras, S. J. Patrick and J. C. Waterlow. *A.M.A. Arch. Int. Med.* 101: 67, 1958.
28. Faber, M. *Acta med. scandinav.* 114: 59, 1943.
29. Reinhold, J. G., L. G. Tourigny and V. L. Yonan. *Am. J. Clin. Path.* 23: 645, 1953.
30. Harrison, M. F. and L. M. Brown. *Biochem. J.* 48: 151, 1951.
31. Grob, D., J. L. Lilienthal, A. M. Harvey and B. F. Jones. *Bull. Johns Hopkins Hosp.* 81: 217, 1947.
32. Wescoe, W. C., C. C. Hunt, W. F. Riker and I. C. Litt. *Am. J. Physiol.* 149: 549, 1947.
33. Brauer, R. W. and M. A. Root. *J. Pharmacol. & Exper. Therap.* 88: 109, 1946.
34. McArdle, B. *Quart. J. med.* (N.S.) 9: 107, 1940.
35. Kunkel, H. G. and S. M. Ward. *J. Exper. Med.* 86: 325, 1947.
36. Waterlow, J. C. *West Indian Med. J.* 1L 41, 1951.
37. Srinivasan, P. R. and V. N. Patwardhan. *Lancet* II: 864, 1952.
38. Bauer, J. *Wien. klin. Wchnschr.* 25: 1376, 1912.
39. Mukherjee, K. L. and G. Werner. *J. Lab. & Clin. Med.* 43: 727, 1954.
40. Dean, R. F. A. and R. Schwartz. *Courrier internat. Child. Cent.* 4: 295, 1954.
41. Demaeyer, E. M. and H. Vanderborght. *Ann. Soc. belge Med. trop.* 34: 417, 1954.
42. DeMagalhães Carvalho, J. *O. Hospital (Rio de J.)* 32: 307, 1947.
43. Dricot, C., P. Beheyt and P. Charles. *Ann. Soc. belge Med. trop.* 31: 581, 1956.
44. Perakis, K. and D. Bakalos. *Deutsche med. Wchnschr.* 69: 746, 1943.
45. Glaser, E. M. *Spec. Rep. Ser. med. Res. Counc.* (No. 275) London: Her Majesty's Stat. Off., 1951, p. 236.
46. Jayasekera, H. T. W., B. V. DeMel and H. Collumbine. *Ceylon J. med. Sc.* 8: 1, 1951.
47. Schwartz, R. *J. Clin. Path.* 9: 333, 1956.
48. Unpublished results, Jamaica.
49. Budiansky, E. *O. Hospital (Rio de J.)* 36: 533, 1949.
50. Wroblewsky, F. and J. S. Ladue. *Ann. Int. Med.* 43: 345, 1955.
51. Stephen, J. M. L. and J. C. Waterlow. Unpublished results, 1957.
52. Cove, S., S. Goldstein and F. Wroblewski. *Pediatrics (Springfield)* 20: 584, 1957.
53. Hutchinson, A. O. *Spec. Rep. Ser. med. Res. Counc.* (No. 275) London: Her Majesty's Stat. Off., 1951, p. 226.
54. DeMarco, B. and F. Cusmano. *Lattante* 26: 388, 1955.
55. McCance, R. A., R. F. A. Dean and A. M. Barrett. *Spec. Rep. Ser. med. Res. Counc.* (No. 275) London: Her Majesty's Stat. Off., 1951, p. 135.
56. Kenawy, M. R. *Trans. R. Soc. Trop. Med. & Hyg.* 31: 339, 1937.
57. Gillman, J. and T. Gillman. *Perspectives in Human Malnutrition.* New York: Grune and Stratton, 1951.
58. Trowell, H. C., J. N. P. Davies and R. F. A. Dean. *Kwashiorkor.* London: Arnold, 1954.
59. Gillman, J., C. Gilbert and T. Gillman. *S. African J. Med. Sci.* 12: 99, 1947.
60. Jackson, C. M. *The Effects of Inanition and Malnutrition upon Growth and Structure.* Philadelphia: Blakiston, 1925.
61. Waterlow, J. C. *Proc. Roy. Soc. Med.* 40: 347, 1947.
62. Davies, J. N. P. *Lancet* I: 317, 1948.
63. Hartz, P. H. *Documenta Neerl. et Indones. Morbis Trop.* 1: 41, 1949.
64. Bras, G. and K. P. Clearkin. *Documenta Med. Geograph. et Trop.* 6: 327, 1954.
65. Bras, G., J. C. Waterlow and E. Depass. *J. Trop. Pediat.* 2: 147, 1956.
66. Véghelyi, P. V. *Ann. paediat. (Basel)* 175: 349, 1950.
67. Véghelyi, P. V. *Acta paediat.* 36: 483, 1948.
68. Badr El-Din, M. K. and M. H. Aboul Wafa. *J. Trop. Pediat.* 3: 177, 1957.
69. Carvalho, M., M. M. Schmidt and A. G. Pinto. *J. de Pediat. (Rio de J.)* 13: 141, 1947.
70. Thompson, M. D. and H. C. Trowell. *Lancet* I: 1031, 1952.
71. Gómez, F., R. Ramos-Galván, J. Cravioto and S. Frenk. *Pediatrics (Springfield)* 13: 548, 1954.
72. McDougall, C. *Pediatrics (Springfield)* 5: 114, 1950.
73. Matsaniotis, N. S. *J. Pediat.* 51: 267, 1957.
74. Bate, J. G. and U. James. *Arch. Dis. Childhood* 31: 506, 1956.
75. Haanes, M. L. and P. György. *Am. J. Physiol.* 166: 441, 1951.
76. Cravioto, J. *Mod. Probl. Paediat.* 2: 169, 1957.
77. Waterlow, J. C. and V. G. Wills. *Nitrogen and Phosphorus Metabolism in Malnourished Infants.* To be published, 1959.
78. McKenzie, A. *E. African Med. J.* 17: 30, 1940.
79. Heard, C. R. C. *Proc. Nutr. Soc.* 16: xi, 1957.
80. Waterlow, J. C. and G. Bras. *Brit. M. Bull.* 13: 107, 1957.
81. Billing, B. H., H. J. Conlon, D. E. Hein and L. Schiff. *J. Clin. Invest.* 32: 214, 1953.
82. Waterlow, J. C. and T. Weisz. *J. Clin. Invest.* 35: 346, 1956.
83. Mukherjee, K. L. and N. K. Sarkar. *Brit. J. Nutrition* 12: 1, 1958.
84. Waterlow, J. C. and S. J. Patrick. *Ann. New York Acad. Sc.* 57: 750, 1954.
85. Waterlow, J. C. *Acta Unio internat. contra cancrum* 13: 704, 1957.
86. Burch, H. B., G. Arroyave, R. Schwartz, A. W. Padilla, M. Behar, V. Viteri and N. S. Scrimshaw. *J. Clin. Invest.* 36: 1579, 1957.
87. Seifter, S., D. M. Harkness, L. Rubin and E. Muntwyler. *J. Biol. Chem.* 176: 1371, 1948.
88. Westerfeld, W. W. and D. A. Richert. *J. Biol. Chem.* 184: 163, 1950.
89. Litwack, G., J. N. Williams, Jr., P. Feigel-

SON AND C. A. ELVEHJEM. *J. Biol. Chem.* 187: 605, 1950.
90. SRIRAMACHARI, S. AND V. RAMALINGASWAMI. *Indian J. Pediat.* 20: 1, 1953.
91. ROSENTHAL, O., J. C. FAHL AND H. M. VARS. *J. Biol. Chem.* 194: 299, 1952.
92. SPOSITO, M. AND S. RUSSO-CA *Il Fegato* 2: 274, 1956.
93. HESTRIN, S. *J. Biol. Chem.* 180: 879, 1949.
94. CHRISTIE, G. S. AND J. D. JUDAH. *Proc. Roy. Soc., London. ser. B.* 141: 420, 1953.
95. WATERLOW, J. C. AND J. M. L. STEPHEN. *Further Observations on Enzymes in Human Liver in Malnutrition.* To be published in Proceedings of the World Congress of Gastroenterology, 1958.
96. METCOFF, J., S. FRENK, G. GORDILLO, F. GÓMEZ, R. RAMOS-GALVÁN, J. CRAVIOTO, C. A. JANEWAY AND J. L. GAMBLE. *Pediatrics* 20: 317, 1957.
97. ALLISON, J. B. In: *Symposia on Nutrition.* Springfield: Thomas, 1950, vol. II.
ALLISON, J. B. *Fed. Proc.* 10: 676, 1950.
ALLISON, J. B. AND R. W. WANNEMACHER. In: *Amino-acid Malnutrition*, edited by W. H. COLE. New Brunswick: Rutgers Univ. Press, 1957, p. 1.
98. ADDIS, T., L. J. POO AND W. LEW. *J. Biol. Chem.* 115: 117, 1936.
99. KOSTERLITZ, H. W. *J. Physiol.* 106: 194, 1947.
100. THOMSON, R. Y., F. C. HEAGY, W. C. HUTCHISON AND J. N. DAVIDSON. *Biochem. J.* 53: 460, 1953.
101. WATERLOW, J. C. AND T. WEISS. *J. Clin. Invest.* 35: 346, 1956.
102. ALLISON, J. B. *Am. J. Clin. Nutrition* 4: 662, 1956.
103. ADDIS, T., L. J. POO AND W. LEW. *J. Biol. Chem.* 115: 111, 1936.
104. DEAN, R. F. A. AND R. SCHWARTZ. *Courrier internat. Child Cent.* 4: 295, 1954.
105. SRINIVASAN, P. R. AND V. N. PATWARDHAN. *Lancet* II: 864, 1952.
106. BIGWOOD, E. J. In: *Malnutrition in African Mothers, Infants and Young Children.* (Report of Second Inter-African Conference on Nutrition). London: Her Majesty's Stat. Off., 1954, p. 249.
107. GREEN, D. E., R. L. LESTER AND D. M. ZEIGLER. *Biochim. et biophys. acta* 23: 516, 1957.
108. DEVLIN, T. M. AND A. L. LEHNINGER. *J. Biol. Chem.* 233: 1586, 1958.

PROTEIN REQUIREMENTS IN INJURY AND CERTAIN ACUTE AND CHRONIC DISEASES

STANLEY M. LEVENSON AND DONALD M. WATKIN

Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C., and the National Cancer Institute, Bethesda, Maryland

"Wherefore, I say, that such constitutions as suffer quickly and strongly from errors in diet, are weaker than others that do not; and that a weak person is in a state very nearly approaching to one in disease . . . and this I know, moreover, that to the human body it makes a great difference whether the bread be fine or coarse; of wheat with or without the hull, whether mixed with much or with little water, strongly wrought or scarcely at all, baked or raw. . . . Whoever pays no attention to these things, or paying attention, does not comprehend them, how can he understand the diseases which befall a man? For, by every one of these things, a man is affected and changed this way or that, and the whole of his life is subjected to them, whether in health, convalescence or disease. Nothing else, then, can be more important or more necessary to know than these things." (1)

TWENTY-FIVE HUNDRED YEARS have passed since Hippocrates admonished us to pay close attention to the connection between nutrition and disease. We lately have heeded this advice, but it can hardly be said that we yet have a full understanding of these matters.

The aspects of these matters which we have chosen to present in this paper are to be regarded as a basis for discussion, rather than an attempt to cover completely so vast a topic. Our report will be concerned principally with protein nutrition in adult males; we will not attempt any systematic coverage of the problem in infants, children, women or the aged. And only a passing mention will be made of the important influence of emotional factors on these problems.

The important question whether the protein 'stores' of the 'healthy' man should be raised by high protein feedings as a prophylactic measure against stressful situations has been discussed elsewhere in this symposium; no definite conclusion can yet be made; this matter will be alluded to again later in this paper. Also, it need hardly be mentioned that, although we will

discuss principally protein metabolism and protein requirements, this nutrient cannot be considered alone; it is a part of the total metabolic mixture. And, finally, we should like to make it clear at the start that the nutritional care of the patient, injured or ill, is not an entity unto itself, but is, rather, an integral part of the total care of the patient.

INJURY AND ACUTE INFECTIOUS DISEASE

The rapid nutritional deterioration which so often follows severe injuries or acute infections is one of the dramatic metabolic problems encountered clinically. It was about 75 years ago that systematic investigation of this problem began with a few measurements of nitrogen excretion by patients with typhoid fever; it was about 50 years ago that Coleman, Shaffer and DuBois began their classic studies of metabolic changes in patients with acute and chronic medical illnesses; and it was about 30 years ago that Cuthbertson clearly described the increased urinary nitrogen excreted following fractures. Since then, there have been hundreds of reports describing metabolic changes after injury from which it appears that apparently all metabolites are affected. But we know relatively little about the physiologic consequences of these early chemical changes. How do they affect the clinical course and convalescence of the injured patients? Should attempts be made to modify them? There are conflicting opinions among physicians and surgeons regarding these points because of lack of sufficient concrete objective evidence. We will review some known facts about the mechanism and significance of the metabolic reaction to injury and acute infectious disease, point out some of the gaps in our information, and will make some provisional recommendations for the nutritional care of the acutely ill or injured patient.

There are many similarities in the metabolic responses of previously healthy subjects to acute infectious diseases and injury; it is these similarities which have prompted us to discuss them together. But these responses are not identical; whether the differences are principally qualitative or quantitative cannot yet be decided with certainty. This is due in part to the virtual impossibility in comparing the severity or gravity of the two situations. Is a patient with an extensive third degree burn more severely 'stressed' (from the metabolic and nutritional points of view) than a patient with overwhelming pneumonia? We will discuss this question at more length later.

Patients with typhoid fever were commonly the subjects of metabolic study in the late 19th and early 20th century. "The fever itself is fairly regular in its course and offers a period of rising temperature, a period of level temperature, sometimes two weeks long and a period of falling temperature with wide remissions. Patients with this disease lend themselves well to experimental procedures. They are apathetic and are not worried by the display of apparatus. Their circulatory systems are dependable and cause the investigator little anxiety" (2).

Müller (3) is generally credited as being one of the first to report serious nitrogen losses by typhoid patients; he found that such a patient lost 10.8 gm of nitrogen daily during 8 days of fever, when his food intake was 8.3 gm N and about 1000 calories daily. Interest in the over-all metabolic aspects of acute infectious diseases reached a peak with the classic studies of Shaffer (4), Coleman and DuBois (5) in the first quarter of the 20th century. Cuthbertson (6) had described the characteristic negative nitrogen balances early after fractures in the 1920's, but it was not until the late 30's and early 40's when the threat, and then the occurrence of World War II aroused widespread interest in this problem.

The usual features of the early metabolic response to injury of previously healthy adults may be summarized as follows (3, 7–21): increased heat production; weight loss; negative nitrogen, potassium, sulfur and phosphorus balances; disturbed carbohydrate utilization, tendency to ketosis, retention of sodium, chloride, and water, and alteration in the metabolism of certain water-soluble vitamins. These changes are usually accompanied by involution of lymphoid tissue and thymus, lymphopenia and eosinopenia, leukocytosis, adrenal cortical hypertrophy with associated depletion of cholesterol and ascorbic acid and an increase in certain adrenal medullary and cortical hormones in the blood and urine. Liver function is altered, as evidenced by an increase in sulfobromophthalein retention and a decrease in the rate of conjugation of adrenal steroids. The metabolic reaction to injury, therefore, consists of a variety of chemical and physiologic changes which involve protein, carbohydrate, fat, water, vitamin, endocrine and electrolyte metabolism in almost every organ of the body. These changes are qualitatively similar

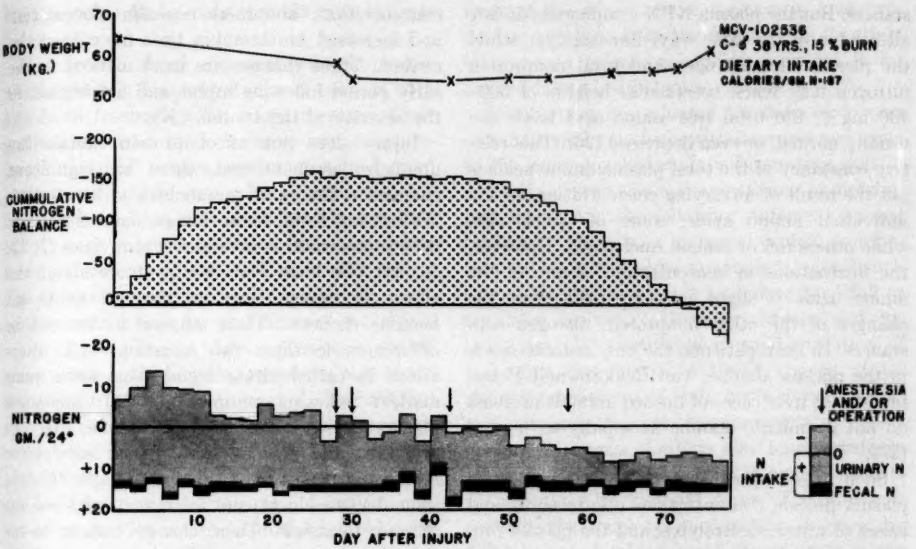


FIG. 1. Body weight and nitrogen balance data of a young man with moderately severe skin burn. In the lower third of graph nitrogen intake is plotted from zero line down, fecal nitrogen in solid black going up from intake line. On top of this is plotted urinary nitrogen. Center section of graph charts the cumulative nitrogen balance.

in patients with a wide variety of injuries or illnesses. The extent of the metabolic imbalance roughly parallels the severity of the injury and is most marked in the previously healthy young adult male. The response is dynamic, with an ever-changing, but generally predictable, pattern. Though all metabolites seem to be involved, the extent and duration of their involvement vary—sodium disturbances are brief; protein changes, prolonged. Finally, with recovery, after variable transition periods, there is a return to normal. Cuthbertson (6) and Moore (22) have reported extensively on these matters. Moore has proposed a theory of convalescence which divides convalescence into four dynamic biological phases which terminate in a patient rehabilitated to health, strength and work. He emphasizes that the duration and correlation of these phases are not rigid, that there are considerable individual variations.

Protein metabolism in particular has been extensively studied. The healthy adult male eating a normal diet is in nitrogen equilibrium. Following injury or acute infection his fecal nitrogen excretion is unchanged (unless there is specific gastrointestinal tract damage), but his urinary nitrogen excretion increases to as much as 45 gm of nitrogen daily in an extreme case. Urea is usually the chief component with proportionately lesser increases of the other urinary nonprotein nitrogen fractions. Proteinuria is minimal in the absence of renal disease or injury.

The increased urinary nitrogen excretion begins soon after injury, reaches a peak at about 5–7 days, and may continue for 3–7 weeks. During this time, the injured patient is in negative nitrogen balance and (fig. 1) there is a gradual depletion of his body protein. As he recovers, his urinary nitrogen excretion falls, and, since his food intake is often high at this time, he goes into positive nitrogen balance. This continues until his body proteins are restored. The early protein depletion is accentuated in patients with extensive surface wounds when the exudate loss is significant, a problem particularly important in burn patients (23, 24). Transient azotemia associated with transient oliguria occurs frequently in patients with injuries of only moderate severity. Prolonged azotemia may occur in severely injured patients who have suffered prolonged severe renal ischemia resulting in acute renal failure. The rises in blood NPN, urea and plasma potassium are considerably faster in these patients than in uninjured patients with acute renal failure (25), probably a result of increased 'production' of nonprotein nitrogenous sub-

stances. But the plasma NPN components do not all behave in the same way. For example, while the plasma urea nitrogen and total nonprotein nitrogen may reach spectacular heights of 300–400 mg%, the total free amino acid levels are usually normal, or even depressed (26). This relative constancy of the total plasma amino acids is not the result of unvarying concentrations of the individual amino acids, some of which rise, while others fall or remain unchanged. However, the fluctuations in concentration of any of the amino acids is slight in comparison with the changes of the other nonprotein nitrogen substances. In burn patients, the only notable rise is in the plasma alanine; van Bekkum and Peters found that liver slices of burned animals in shock do not deaminate alanine as rapidly as normal (27).

Soon after injury there may be changes in plasma protein concentrations due to shifts and losses of water, electrolytes, and the plasma proteins, of which albumin shows the most marked decrease. These changes are most marked in patients with severe burns (28). If shock is severe, there is often a decrease in prothrombin and fibrinogen concentrations (29). Changes in tissue proteins and protein turnover will be discussed in the section 'Source of Increased Urinary Nitrogen Excreted after Injury.'

Late after injury, there may be hypoproteinemia as part of the generalized tissue protein depletion. Again, the albumin fraction is chiefly decreased; the changes in the other protein fractions are variable depending on such factors as the presence of infection and liver function.

Little is known about altered enzyme activity following injury (30, 31). Some of the reported changes are an increase in proteolytic, lipase, and phosphatase activity in the lymph draining a burned area (32, 33), an increase in plasma fibrinolysis in severe shock (29), and a decreased ability of liver slices from burned rats to deaminate alanine or to make glycogen (27, 34). It appears that alterations in many enzyme systems may occur, but considerably more information is needed before the significance of these changes can be established. Similarly, little is known of the effect of trauma on the various nucleoprotein metabolites of tissue and blood (35, 36).

Some disturbance of liver function is not uncommon in extensively injured patients, particularly if shock has been severe. Increased bromsulfalein retention, increased urine urobilinogen excretion, increased serum bilirubin concentration, abnormal cephalin flocculation and increased prothrombin time have been described. These changes are most marked in the early period following injury and are related to the severity of the trauma (37).

Injury does not affect protein metabolism uniquely; as mentioned, there are significant changes in almost all metabolites so far studied. We will not detail these changes since they have been summarized in several recent reviews (7, 22, 38, 39). We know considerably more about the effects of injury, however, than of acute infectious diseases. There appear to be certain differences in these two situations—*e.g.* alterations in carbohydrate metabolism seem more marked following trauma (40, 41); increased plasma pyruvic acid, lactacidemia, hyperglycemia, and acidosis may appear soon after injury and persist over a period of days; there is some decrease in glucose tolerance and some resistance to insulin. These changes appear to reflect an increase in gluconeogenesis and in peripheral carbohydrate breakdown.

The problems of oxygen consumption, carbon dioxide production and heat production of patients with acute infectious disease were investigated fully in the first quarter of the 20th century by Coleman, Shaffer, DuBois and others (4, 5); an admirable summary of these classic studies was presented by DuBois (2); many of our comments are drawn from his account. As expected, they found an increase in total heat production and destruction of protein during the febrile period, but the increase in total metabolism was comparatively slight, not approaching the levels found in severe hyperthyroidism or moderate muscular exercise. The average increase during the periods when typhoid patients showed great toxemia was 23–44%. All the evidence from direct and indirect calorimetry showed that there was no profound change in oxidative processes. Measurements of respiratory quotients showed that patients who are in the early stages of fever and who are on low diets rapidly approach the condition of partial starvation, having practically exhausted their stores of glycogen. The patients who are well-fed seem to differ in no way from normals, but they must be given more food than normals to bring the quotients to the same level. Depleted glycogen stores are rapidly replenished during convalescence, the respiratory quotients rise to 'an extraordinary level' and the metabolism which had been on a protein-fat basis in

fever appears to be on a protein-carbohydrate basis.

Cuthbertson also found an increase in oxygen consumption in his early studies of patients with long-bone fractures (42). More recently, Cope and his associates made multiple brief measurements of 'basal' oxygen consumption by burned patients, beginning soon after trauma and extending throughout convalescence. The majority of severely burned patients increased their metabolic rates to plus 30–60% early after injury. The oxygen consumption rate gradually declined, but continued high for as long as 2 months in many of the patients. Although fever probably plays a role in the elevated metabolic rate of the severely burned patient, Cope *et al.* observed that the temperature rises in the patients were irregular and did not correlate with the metabolic rate (43).

They speculate that the rate of oxygen consumption may be increased in the injured areas due to heightened cellular and chemical activity, and that it is this increase in local activity which in part accounts for the increased metabolic rate of the severely injured patient. In this regard, they point out the correlation of the increased over-all metabolic rate with the extent of the wound area in the burned patients. However, specific information on the actual metabolic rate of the injured areas is not available. Muus and Hardenberg (44) have observed that the oxygen consumption of normal rat liver slices is increased when incubated in lymph from the burned legs of calves and dogs.

Cairnie (45) and his colleagues have recently studied the increase in total metabolism of rats given constant high-protein diet (20% protein), as measured in a gradient layer calorimeter. They found that the metabolism rapidly rose to a peak on the 3rd day after fracture and then gradually declined, a course which paralleled the characteristic curve of urinary nitrogen excretion. The heat production due to protein catabolism was calculated from the urinary N excreted by rats with fractures, these rats having been caged outside the calorimeter under the same dietary conditions. (The calculation assumed that the nonprotein residues were oxidized for energy.) When this quantity was set alongside the data for the total metabolism, close agreement was found both in the magnitude and in the duration of the observed and calculated increases in heat production. On the evidence of this close agreement, Cairnie and his associates feel that following fracture the whole of the increase in heat production resulting from the fracture has its origin in increased protein catabolism, and not in increased fat and carbohydrate catabolism.

Cuthbertson re-evaluated his data of oxygen consumption by patients with fractures and came to the same conclusion. It is interesting that in 1903, May summarized the conditions of metabolism in fever in the following statement quoted by Lusk: "There is an increased proteid metabolism but no increased fat metabolism, except such as may incidentally result from dyspena, chill or muscular activity." However, Moore and his associates (46) postulated a rapid oxidation of body fat after injury, a result arrived at by indirect estimations of body fat from body water and nitrogen balance measurements of postoperative patients. DuBois also found evidence of increased fat oxidation from RQ measurements of patients with typhoid fever. The apparent contradiction may be explainable, in part, by differences in food intake of the subjects in the several studies.

Injury may give rise to abrupt disturbances in water and electrolyte metabolism (10, 46, 47). For example, the early migration of fluid into, adjacent to, and from the injured area have long been recognized. There may be a general increase in measured extracellular space. Serum sodium and chloride concentrations drop in the early postinjury period; there is a shift of sodium into injured cells with a concomitant outward shift of potassium. Sodium and chloride excretions are initially low, resulting in early positive balances, but later, during the period of resorption of wound edema and diuresis, these balances transiently become negative. Generally, the periods of these changes are considerably shorter than those of the changes in protein metabolism. Potassium balance is usually negative for the early days after injury but positive balance is gradually obtained as potassium intake is recommenced. Apparent correlation between nitrogen and potassium balances is variable—there is sometimes a greater early potassium loss than would be expected from the known potassium to nitrogen ratio in normal tissue. Also, potassium balance may become positive sooner than nitrogen balance.

Less is known regarding other electrolytes. The few studies of sulfur metabolism have indicated a close relationship to protein. Increased urinary excretion of calcium may occur early after injury, particularly if bones have been fractured

(48, 49). Decreases in plasma ascorbic acid concentration, its urinary excretion (not followed by a period of increased loss), and 'tissue saturation' as measured by intravenous 'load tests' have been described early after injury. The more severe the injury, the greater the disturbances, with the changes greatest in the early period following injury, but sometimes continuing far into convalescence (50–52). Urinary excretion and 'tissue saturation' of thiamine and nicotinamide also decrease after injury. Riboflavin excretion also decreases early after injury, but is sometimes followed by a brief period of increased excretion. During the convalescent period, the patient's positive nitrogen balance is accompanied by a positive riboflavin balance. We know nothing about the effect of injury on the fat-soluble vitamins.

Source of Increased Urinary Nitrogen Excretion after Injury. Cuthbertson (53), in his early studies in the 1930's concluded that the nitrogen loss after injury "was probably more general than local"; this thought was based on the magnitude of the loss, as in one fracture case 137 gm N, equivalent to about 4.38 kg of muscle, were lost in the course of 10 days. More recently, Madden (54) using S^{35} labelled methionine, has investigated the effect of turpentine abscesses on the urinary excretion of sulfur and its incorporation into the plasma protein and various other tissue proteins of dogs. The tagged methionine was given one day after injection of the turpentine; the dogs were killed 24 hr later. There was an increased output of both sulfur and nitrogen as compared with control dogs, but no increase in urinary excretion of the S^{35}. The build-up of S^{35} specific activity in various tissue proteins was generally comparable in the abscessed and normal animals, though there was a distinctly higher activity in the plasma protein of the abscessed dogs. Levenson, Crowley, Braasch and Mueller (55) also studied this problem using rats. They

TABLE 1. NITROGEN METABOLISM IN CONTROL AND BURNED RATS

Atom % Excess N^{15}

Organ	Unburned	Burned	p Value
Skin	0.031	0.029	0.7
Carcass	0.031	0.032	0.7
Heart	0.050	0.058	0.01
Adrenal	0.086	0.108	0.001
Kidney	0.097	0.123	0.01
Liver	0.116	0.142	0.01
GI tract	0.139	0.169	0.05
Plasma protein	0.236	0.426	0.001

TABLE 2. PLASMA PROTEIN

Atom % Excess N^{15}

Day	Unburned	Burned	p Value
0	0.319		
1	0.258	0.205	
3.5	0.146	0.119	0.001
6.5	0.103	0.083	0.01

TABLE 3. 6.5 DAYS AFTER ANESTHESIA, OR ANESTHESIA AND BURN

	Body Wt., *gm*	Carcass Wt., *gm*	Carcass Prot. N, *gm*	Carcass Prot. N^{15} *mg*
Unburned	321	196	7.00	3.06
Burned	296	168	6.10	2.35
p Value	0.05	0.001	0.02	0.001

wanted to find out what tissue proteins are involved in the metabolic response to a burn, to what extent, and whether the increased urinary nitrogen excretion reflected heightened tissue breakdown or decreased tissue build-up. They fed N^{15} glycine at predetermined times to young adult rats, burned and unburned, receiving a constant diet by gastric tube sufficient to maintain the unburned rats in slight positive nitrogen balance. In one set of experiments, a tracer dose of the N^{15} glycine was given at the height of the period of negative nitrogen balance; the rats were killed 12 hr later. In a second set of experiments, the N^{15} glycine was given daily for 4 days just prior to burning of the experimental group; the rats were then killed 1, 3½ and 6½ days later. The incorporation of N^{15} in the control animals of first experiment was least in the skin and carcass protein and greatest in the liver, gastrointestinal tract, and plasma proteins (table 1). In the burned animals, the same general order of incorporation was observed. In each case, the N^{15} incorporation in the tissue proteins of the burned animals was equal to, or greater than, that in the corresponding tissues of the control rats. If we assume that incorporation of N^{15} in the tissue proteins signified synthesis, then anabolism was occurring in the burned rats and the increased urinary nitrogen excretion cannot be solely explained on the basis of anti-anabolism.

Table 2 lists the plasma protein data of the second experiment; the decline of the N^{15} concentration was faster in the burned rats. It was also found that the protein content of the very active organs such as the liver, changed little despite their markedly increased turnover rates in the burned rats. By contrast, the protein content of the carcass fell, despite a much slower turnover

rate, and, indeed, accounted mathematically for most of the extra urinary nitrogen loss (table 3). This suggests that the integrity of certain vital organs is maintained at the expense of less active areas, such as the skeletal muscle. Moore (22) has also proposed from clinical observations that the skeletal muscle is the major source of the extra-urinary nitrogen excreted after injury. Some of the increased urinary nitrogen may come from autolysis and absorption of the directly injured tissue.

These isotopic experiments suggest that both increased anabolic and catabolic processes go on in the injured rat and dog, with catabolism predominating; all tissues do not participate equally in this response. Presumably, man responds to injury in the same way, but the available information is very limited. Blocker and his associates (56) observed that S^{35} labeled l-methionine is incorporated at an accelerated rate into the serum proteins of patients during the early period following burns, which they interpret as indicative of increased anabolism. At the same time, though, they state that catabolism is going on at an even greater rate with the net result of an increased urinary nitrogen excretion. Sterling, Lipsky and Freedman (57) found that I^{131} tagged albumin disappeared from the circulation of surgical patients in the early postoperative period faster than in normal control subjects but "the failure to recover radioactivity in the urine in amounts comparable with the rapid plasma decline suggests there may not necessarily be a greatly increased rate of complete degradation of albumin to free amino acids. . . . It seems reasonable to suggest the possibilities of incomplete degradation of albumin, or a redistribution or enlargement of the 'albumin space' in the injury reaction If the latter explanation were correct, the rapid falls of plasma radioactivity would represent prolongations of the distribution phase."

MECHANISM OF EARLY CHANGES IN PROTEIN METABOLISM FOLLOWING INJURY AND ACUTE INFECTIOUS DISEASES

Fever, Increased Oxygen Consumption, Severity of Injury or Acute Infectious Disease. The following problem arose early in the investigations of protein metabolism in patients with infectious disease: did their increased temperatures during the acute periods of their diseases account for their increased nitrogen loss? Pflüger, in 1878 (58), had shown that oxygen consumption of normal rabbits increased about 11% and their carbon dioxide production to about 14% when their rectal temperatures were raised from 38.6° to 40.6° by exposing them to external heat. Lusk cites a 1904 report by Linser and Schmid (59) about two men suffering from ichthyosis hystrix which invclved almost complete loss of function of their sweat glands. "The body temperature of these men could be varied by altering the temperature of their living room between 30° and 38° C. The humidity of the room was from 40 to 50 per cent. The maximum increase in the metabolism of these individuals is represented by a rise in carbon dioxide excretion from 3.8 cc. per minute and kilogram at the body temperature of 36.2° to 5.3 cc. per minute and kologram at 39°. The total increase in the carbon dioxide output, due to a rise of 3° through simple warming of cells amounted to 40 per cent." They also found that an increase of body temperature to 39° did not change nitrogen excretion, but above this, urinary nitrogen increased. They concluded that "in toxic fevers where the temperature is not above 39° any increase of protein metabolism must be due to the toxic processes and not to the hyperthermia." This may be the origin of the term 'toxic destruction of protein' which appeared so commonly in the medical literature until the middle 1904's. Further evidence was obtained by dietary experiments that the 'protein destruction' found in patients with acute febrile diseases was not the result of the fever alone. Carbohydrate added to the normal diet prevented the increase in urinary nitrogen excretion by normal subjects whose temperature was raised by exposure to heat, but it had less effect in patients with typhoid fever (von Leyden and Klemperer (60) or paratyphoid fever (Kocher (61)). Shaffer and Coleman (4), and Coleman and DuBois (5) also found that considerably more food calories than their measured heat production had to be given to patients febrile with typhoid fever in order to achieve nitrogen equilibrium. More recently, Grossman and his associates (62) found that negative balances persisted in patients with meningococcus meningitis after the temperature had become normal and symptoms and signs of the disease had been abolished by drug therapy.

Fever is not responsible for the major protein loss following injury; many patients with very little rise in temperature, or no rise at all, show the characteristic increase in urinary nitrogen excretion after injury.

There is clearly a close connection between the

TABLE 4. CHART SHOWING NEGATIVE NITROGEN BALANCES IN TYPHOID PATIENTS WHO RECEIVE FOOD CALORIES IN EXCESS OF CALCULATED HEAT PRODUCTION [DUBOIS (2)]

Patient	Dates or Days of Disease Inclusive	Days in Period	Range of Max. Temp., °F	Calc. Heat Production, Cal*	Food Cal*	Food N Gm*	N Bal., Gm*
Morris S.	Oct. 23–Nov. 3	12	102.8–104.6	2,266	2,863	16.4	−4.4
	Dec. 19–24	6	101.9–105.1	2,085	2,989	13.2	−2.4
Charles F.	Nov. 28–30	3	101.2–103.4	1,752	2,458	12.0	−4.6
Karl S.	Jan. 12–18	7	101.0–105.0	2,197	2,985	16.1	−3.2
	Jan. 19–22	4	98.8– 99.0	1,678	2,819	14.6	−1.9
John K.	Jan. 15–20	6	103.2–104.0	2,568			
	Days of Disease						
Frank W.†	11–14	4	104.0–105.4	2,200	2,250	11.3	−5.0
	15–19	5	103.0–104.0	2,238	3,320	15.3	−3.3
	20–23	4	101.0–103.6	2,054	2,362	15.9	−1.5

* Figures given are averages for 24 hr.
† From Coleman and DuBois, 1914.

severity of the disease or injury and the degree of resulting metabolic imbalance; the greater the injury, the greater the response. Moore and Howard have suggested a classification for grading the severity of uncomplicated traumatic and operative injuries; the reader is referred to their writings (8, 22, 63). Shaffer, Coleman and DuBois, as well as Grossman *et al.*, have pointed out that it is the 'gravity' of the acute infection which determines the intensity and duration of the metabolic response. But how quantitate the gravity of the illness? At the moment, this can at best be a rough clinical estimate.

The critical role of the severity of the stressor situation in the ensuing metabolic changes is illustrated by the recent study by Watkin and Steinfeld (64). They studied the effects of pharmacological doses of corticosteroids given over periods of 1–3½ wk to 6 patients with neoplastic disease; a summary of some of their findings appears in table 6. Data collected on 2 of the patients appear in figures 4 and 5. After suitable control periods on constant diets, the patients were given prednisone in doses ranging from 50 to 100 mg/day to one man and 100 to 1000 mg/day to 3 other men and 2 women for periods ranging from 8 to 24 days. The design of the studies called for each patient to ingest the maximum tolerated quantity of calories and protein during the control phases. No attempt was made to increase the calorie or protein intake during the periods of prednisone administration although all patients demonstrated an increased appetite. Because of marked differences in body weight and appetite among individual patients, appreciable differences in calorie and protein intake both on total body wt and on a per kg basis existed within the group.

Among the 6 patients the diets contained from 50 to 112 gm protein/day or from 0.80 to 1.72 gm protein/kg body weight/day and from 1650 to 3500 calories/day or from 30 to 54 calories/kg body weight/day; as mentioned, the diet remained constant for each patient. The nutritional status (based on a clinical appraisal of the patient from the standpoint of body weight, duration, extent and activity of neoplastic disease, physical strength, degree of cachexia, presence of anorexia and degree of anemia) ranged from excellent to poor.

There was a direct relationship between the negative N balance, weight loss and the mg prednisone administered. Despite the differences in the patients' ages and sexes, in the types and durations of the neoplastic process in the variable protein and calorie intakes and in the nutritional states of the individuals there was remarkable uniformity in the response of these patients to the standard stress of large doses of prednisone.

Considerations of the N/P ratios derived from the balance data suggested to Watkin and Steinfeld that the source of N was tumor only in the patients with susceptible lymphoma and then only early in the course of the treatment. In other patients and in the patients with lymphoma later in their course, the source of the nitrogen seemingly was normal protoplasm, presumably muscle.

Although no specific studies of antibodies were performed, some patients did develop pancytopenias and all were plagued by a variety of bacterial and fungal infection, thromboses,

emboli and in one instance a large intramuscular hematoma with subsequent tissue necrosis and infection. The impression arising from these experiences was that there was progressive protein depletion and that associated with this depletion resistance to bacterial and fungal infections and to physical trauma was reduced.

Effect of age, sex, nutritional status, recent preceding injury. It is the well-nourished, healthy, young men who show the greatest urinary nitrogen excretion after injury; significantly less protein is lost by the woman, the child [but apparently not after acute infectious disease] or the undernourished patient. Browne (65) noted that the metabolic response to injury is lessened when the patient is in the 'anabolic' phase after an earlier injury. Howard (66) noted a progressive decline in the increased urinary nitrogen excretion following malarial chill and fever episodes, recurring at close intervals. Why these facts are so is not clearly understood. Munroe and his colleagues (67) found that rats fed a protein-free diet until they were markedly depleted failed to develop the usual negative nitrogen balance and increased heat production after femoral fractures. They have postulated the existence of 'storage' protein and that this is the protein which is lost following injury. According to this hypothesis, if storage protein is depleted prior to injury, no increase in excretory nitrogen would occur after severe injury. Others have written in a similar vein (68, 69).

Thyroid. Several investigations of thyroid function after injury have been reported; these were prompted by the already mentioned phenomenon of increased heat production by injured patients (43, 70–72). The presence of active thyroid hormone is probably necessary for the usual increased nitrogen loss, but evidence regarding increased thyroid activity in this circumstance is controversial. Our interpretation is that while there may be a very brief increase in thyroid activity following trauma, this increase is neither intense nor prolonged enough to account for the protein loss.

Central Nervous System, Hypothalamus, Pituitary and Adrenals. There is clear evidence that the metabolic reaction to injury may be mediated in part through the neuro-endocrine system. Early impetus for this view came from Claude Bernard, Walter Cannon and Hans Selye. The state of adrenal function was noted to influence profoundly the capacity of the organism to respond to stress. Early studies showed that adrenal medullary activity normally increases abruptly following injury (73, 74). Selye (75) later reported tissue distintegration, cellular atrophy, and even necrosis in various tissues, with the notable exception of the adrenal cortex. This organ, on the contrary, almost invariably hypertrophied. These histologic changes in the adrenal have since been correlated with an early increased secretion of certain adrenal cortical steroids as measured by increases in urinary and blood steroid products (76–79).

A relationship between the responses of the pituitary and adrenals was also demonstrated in the early stress studies. It was known by 1920 that adrenal cortical atrophy occurred after hypophysectomy. Selye (80) predicted and showed that hypophysectomy, like adrenalectomy, prevented the usual reactions to stressing stimuli. Extension of these observations led Selye to formulate his concept of the 'general adaptation syndrome.' Later observations showed that ablation or stimulation of certain areas of the hypothalamus either depresses or activates the secretion of corticotropin (ACTH) (81–83). Apparently the hypothalamus activates the anterior pituitary humorally. There has been much discussion about how the hypothalamic-pituitary-adrenal system is activated. Both nervous and metabolic factors are involved.

Since the concepts of the general adaptation syndrome and, more recently, the local adaptation syndrome have been discussed so frequently and fully elsewhere, they will not be elaborated here. Almost all metabolites are affected by the various pituitary and adrenal hormones, though the precise manner in which these hormones modify metabolic reactions is not known. After injury, certain adrenal hormones are apparently secreted in amounts that are capable of causing metabolic upsets if given to otherwise normal subjects. Further, as already mentioned, many of the biochemical responses following injury seem to be related to the presence of an injury of a certain severity rather than to one of a specific type and, in this sense, seem nonspecific. Since many of these metabolic changes are similar to those induced by excess adrenal hormones, increase in adrenal activity has been considered important in the metabolic reaction to injury. This thesis has gained some support from the findings that (1) many of these changes do not occur in the unsupported adrenalectomized animal and (2) some of the metabolic changes and the apparent adrenal cortical hormone excretion

roughly parallel the severity of the injury. However, certain studies have demonstrated the occurrence of some of the biochemical changes associated with injury in the presumed absence of any concomitant increase in adrenal hormone production (84–86).

Ingle (87) has called this the 'permissive' or supporting' action of the adrenocortical hormones; Selye (88), from a different point of view, speaks of the 'conditioning' action of these hormones. But is the physiologic effect of exogenous adrenal cortical hormone constant when it is infused at a constant rate in the injured animal? Current evidence suggests that certain responses to the adrenal steroids are modified by associated systemic stress (89). This may result from an increase in the blood concentration of the 'free' hormone as a result of a decrease in conjugation of circulating steroids by the liver (90) or there may be a change in target tissue reactivity. It is generally accepted that there is an association between the extent of metabolic response and the level of cortical hormones secreted. The level of adrenocortical hormones which are necessary for the full metabolic reaction of adrenalectomized animals depends on the severity of the injury. The specific nature of the relationship between biochemical changes resulting from injury and adrenal activity is not known. Studies of specific tissue metabolism will probably be required to establish this. It should be pointed out that the period of demonstrable increase in steroid output is considerably shorter than the period of increased urinary nitrogen as excretion.

Considerably less information is available regarding the behavior of the other endocrine glands after injury. There is an increased secretion of antidiuretic hormone (ADH) (91), which doubtless affects renal function. ADH may be one of the mechanisms whereby the secretion of corticotropin is stimulated.

Physical Inactivity of the Patient. A few investigators have attempted to differentiate between the effects of bed rest per se and the effects of injury or disease. Dietrick and his associates (92) studied healthy young men before, during and after a prolonged period of bed rest. The subjects were given a constant diet calculated to keep them in caloric, mineral and nitrogen balance. Activity was minimized by immobilization of the pelvic girdle and legs in plaster casts extending from the umbilicus to the toes. During the bed rest and immobilization period of 6–7 wk, there was an average loss of about 54 gm of nitrogen, and losses of calcium ranging from 9 to 24 gm; there was an increase in the excretion of phosphorus, total sulfur, sodium and potassium. The recovery or return, of metabolic functions to control levels was slow.

The quantity of nitrogen lost from immobilization is small when contrasted with that lost as a result of starvation, but still accounts for a significant proportion of the total nitrogen lost (93) by injured patients on a 'normally adequate' diet.

Amino Acid Imbalance. The possibility was suggested a number of years ago that a disturbance in the metabolism of some of the amino acids may be responsible for the protein loss following injury (94). However, there is no conclusive evidence substantiating this view (95, 96).

Effect of Food Intake on the Nitrogen Loss After Injury and Acute Infections. Voluntary food intake is usually low early after injury or early in the course of acute infectious diseases. These patients usually have a poor appetite and their gastrointestinal tract function may be impaired. It has been clear from the earliest metabolic studies of these patients that their nurtriture was 'conditioned' in large part by their food intake.

Holden, Abbott and their associates (97, 98) found that the early nitrogen and weight loss after mild or moderate operative trauma in patients receiving only limited amounts of glucose solution as the exogenous caloric source were similar to that observed in healthy, unoperated volunteers on similar nutrient intakes. Attention should also be directed to the thoughtful studies of Wilkinson (99) of patients kept on almost totally limited food and water intakes during the early postoperative period following major surgery with good clinical results; his continuing studies will be watched with interest. Holden (97) has stated: "The major portion of the nitrogen deficit reported to be the result of operative trauma, in fact, is the result of poor nutritional intake." But this is not true of a patient who has had a severe accident or of one seriously ill with an acute infectious disease; the nitrogen lost by these patients is greater than that lost by healthy subjects on comparable diets, including those losses probably due to bed rest and fever (100). This is so when the diets are low in both protein and calories, low in protein but not in calories, 'normal' in protein and calories, and finally, high in protein and calories. But the net nitrogen loss is less when the food intake level is kept high than when the intake is low (101, 102) (fig. 2).

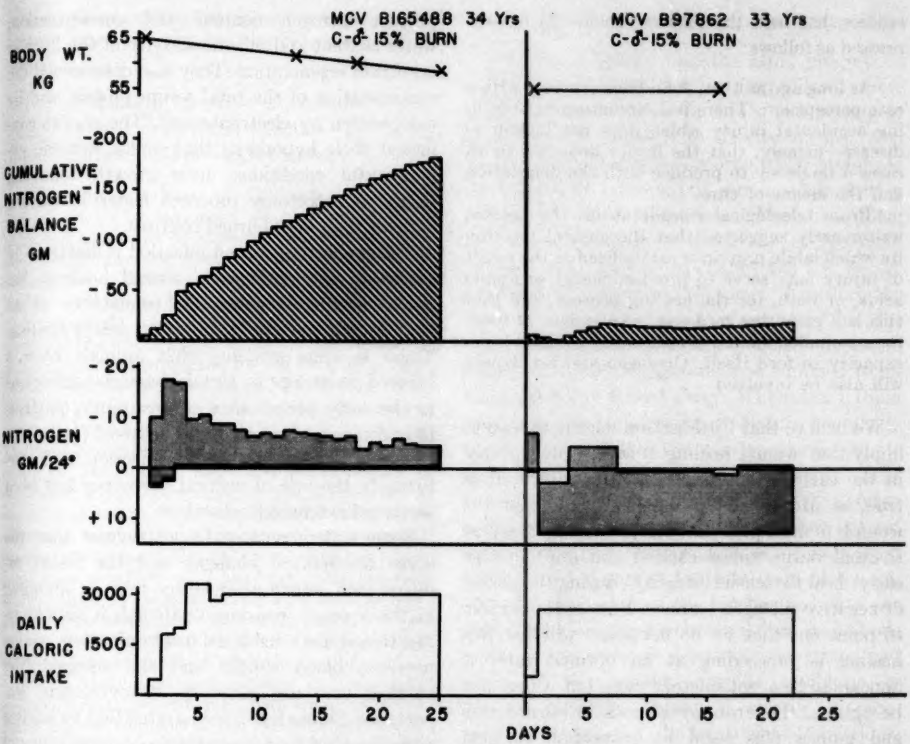


FIG. 2. Nitrogen metabolism following burns: effect of protein intake. *Left*, Pt. (MCV B165488). This 34-year-old Negro, previously in good health, was admitted a few hours after receiving 3rd-degree flame burns involving 13% of his body surface and second-degree burns involving 2%. His burns were washed and dressed with dry occlusive dressings. No parenteral therapy was given. He drank 0.45% saline the first day and milk the second day. From day 3–26, he drank a liquid feeding providing daily 45 cal/kg of body weight, but no protein. Forty % of the calories were provided by fat, 60% by carbohydrate. The diet was well-tolerated. During this time he was in negative nitrogen balance, and lost about 175 gm nitrogen. Except for weight and nitrogen loss, his course was uneventful. While still on the same intake, initial skin grafting was successfully carried out on day 27 and the donor sites healed promptly. *Right*, Pt. (MCV B97862). This 33-year-old Negro, previously in good health, was admitted a few hours after receiving 3rd-degree flame burns involving 13% of his body surface and 2nd-degree burns involving 2%. His burns were washed and dressed with dry occlusive dressings. Fluid therapy during the first day consisted of dextran, saline and water. On the second day, oral liquid diet providing 1.5 gm of protein and 45 cal/kg of body weight was begun and maintained at this level for the next 7 wk. The daily total dietary intake was 82 gm of protein and 2500 calories. Twenty per cent of the calories were supplied by fat. This diet was well-tolerated; the period of apparent negative nitrogen balance was short, and the quantity of nitrogen lost was minimal. Body weight was unchanged. His course was uneventful; skin graftings were successfully carried out on days 16, 23, 35, and 60. He began walking on day 44 and was discharged on the 92nd hospital day. (Reproduced from Lund and Levenson (103).)

WOUND HEALING AND INFECTION

It is clear, then, that the degree of protein lost after injury or acute infectious disease can be lessened by dietary means. Is this desirable? When we consider what the food intake of the severely injured patient should be, we are immediately faced with the fact that little is known concerning the clinical and physiological significance of the characteristic biochemical changes. But there is little doubt that if the metabolic imbalance persists uncorrected for an unduly long time, serious malnutrition will result (103, 104). But what of the early postinjury period, when nitrogen loss is at its height, before overt malnutrition is evident? Cuthbertson, since his early

studies, has held the view which he (7) has expressed as follows:

"As long ago as 1794, John Hunter wrote with a rare perception: 'There is a circumstance attending accidental injury which does not belong to disease—namely, that the injury done has in all cases a tendency to produce both the disposition and the means of cure.'

"From teleological considerations the present writer early suggested that the general reaction by which labile protein is catabolized as the result of injury may serve to provide energy or amino acids, or both, for the healing process, and that this is a primitive response independent of food, for a wounded animal is necessarily reduced in its capacity to feed itself. Glycogen and fat depots will also be involved. . . ."

We believe that Cuthbertson does not mean to imply that wound healing is necessarily optimal in the early catabolic period after injury. It is true, as Moore (22) has pointed out, that the wounds of most patients (except those with severe thermal burns unless excised and grafted very early) heal to 'tensile integrity' during the period of negative nitrogen balance. But we should like to point out that we do not know whether this healing is proceeding at an optimal rate; it appears to be a 'satisfactory' rate, but it may not be optimal. Laparotomy wounds in burned rats and guinea pigs seem by inspection to heal normally, but histologic observations reveal significant delays (105–107). May this not be the case in man? We are currently investigating this at the Walter Reed Army Institute of Research. As mentioned in the section "Source of the Increased Urinary Nitrogen After Injury," it would seem that the integrity of the liver of the burned animal is maintained at the expense of the carcass. If this is so, one would expect that the healing of liver 'wounds' in burned animals would not be slower, but perhaps faster than in unburned animals. This would be in distinct contrast to the behavior of the carcass, or laparotomy wound. This hypothesis was also suggested by experiments of Glinos (108) indicating that depletion of plasma proteins through plasmapheresis initiates active growth of the intact liver; it will be recalled that hypoproteinemia and hypoalbuminemia are characteristic of severely burned animals.

Rather than studying the healing of liver wounds with its attendant problems of bleeding and bile leakage, Levenson *et al.* (109) followed liver growth and regeneration after partial hepatectomy. They measured the total weight, protein nitrogen content and concentration, water content and mitotic activity of the liver as criteria of regeneration. They also determined the concentration of the total serum protein and its composition by electrophoresis. The results confirmed their hypothesis that under suitable experimental conditions, liver growth following partial hepatectomy proceeds faster in burned animals than in unburned controls.

The problem of wound infection is inextricably bound to the problem of wound healing; impaired healing increases the probability of infection and, conversely, infection delays healing. There is some evidence that animals have a lowered resistance to certain bacterial infections in the early period after severe injury, particularly if shock is present or has occurred (110, 111). The role of the adrenals in infection and particularly the role of cortical hormones has been discussed extensively elsewhere.

Some experiments of Dubos suggest that the early biochemical changes and the usual reduced food intake after injury may be involved in the lowered 'resistance'; though it should be mentioned that bacterial contamination, tissue necrosis, blood supply and the adequacy of surgical care are generally of overriding importance. Dubos has demonstrated that an abrupt withdrawal of food increases the susceptibility of mice to experimental infections (112–114).

"Albino mice were fed *ad libitum* diets differing in their contents in proteins, amino acids, and fat, but providing adequate caloric and vitamin intake. At different periods of time after being placed on the experimental diets the animals were infected with standardized dose of either one of three infectious agents: *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Mycobacterium fortuitum*.

"It was found that profound differences in susceptibility could be elicited by changes in the dietary regimen, even when the latter did not affect the weight curves of the noninfected animals. For example, mice fed commercial pellets (which are adequate for growth) were much more susceptible to Staphylococcus than were mice receiving a diet containing 20 per cent casein. A great increase in susceptibility to the three infections occurred when the casein content of the diet was reduced from 20 to 9 per cent, even though the uninfected animals grew well on the latter diet. When the complete diet was replaced by one containing gelatin as sole source of protein, the increased susceptibility took place within 2 days; normal resistance could be restored rapidly by adding casein in proper amount.

"Preliminary results indicate that the fat content of the diet is of great importance in deter-

mining the response of mice to experimental tuberculosis, but not to Staphylococcus infection."

The mechanism for these alterations in susceptibility to experimental infections is not yet known. Dubos (115) previously stated:

"Through a number of different mechanisms arrived at by evolutionary adaptation, man has achieved some sort of ecological equilibrium with the microorganisms that are ubiquitous in his environment. Indeed, these microorganisms may be harbored in the body without causing disease, and establish relationships with the tissues—covering the whole gamut from the commensalism and symbiosis represented by the flora of the gut, to the various types of arrested or latent infections. The factors which determine whether the microbial agents are thus held in check, or undergo unrestrained multiplication, are of course highly complex. Metchnikoff had identified resistance to infection with the ability of the leucocytes to engulf and destroy infectious agents. It seems now that this mechanism, even though supplemented by various humoral factors, operates in but a very few types of infections. I have presented the view that natural and acquired resistance depends more often upon the ability of the inflammatory response to create in and around the lesion a microenvironment inimical to the infectious agent.

"It is likely that disturbances in the general state of health often bring about qualitative and quantitative changes in the biochemical characteristics of the inflammatory area. These in turn may interfere with the processes which control the activities of micro-organisms within the lesion. As a result, the response of the body to infection and consequently the micro-environment in which the infectious process follows its course, are under the control of factors which may be metabolic or psychic in origin. This concept accounts in part for the fact that susceptibility to infection can change independently of the immunological state of the infected individual. Since changes in susceptibility can occur rapidly, and be extremely transient the intensity of exposure to an infectious agent may be less decisive than the physiological state of the exposed individual, determining whether infection fails to take hold, becomes established, runs an abortive course, or evolves into overt disease."

There is no clear-cut evidence that the resistance to infection in the seriously injured patient is decreased. However, only limited information is at hand. Balch (116) found no change in the phagocytic properties of peripheral leucocytes of soldiers who had recently been severely wounded. He also found no decrease in the rise of antibodies following injection of booster doses of tetanus toxoid into these soldiers. Further observations are clearly needed.

NUTRITIONAL PROPHYLAXIS AND THERAPY

There is no doubt that the rapid progressive nutritional depletion that so often follows severe acute infectious disease or injury can be largely prevented by appropriate dietary means. And there is little doubt that morbidity is prolonged and mortality increased if serious malnutrition complicates these conditions. Logically, then, nutritional prophylaxis should be energetically pursued since clinical experience has not wholly substantiated Galen's (117) view: "For since the substances of all animals are in perpetual flux, the whole body will be thus destroyed and dispersed, unless other similar substances be supplied to replace what has flowed away. Wherefore I think that from the very beginning Nature has given, not only to animals but also to plants, innate appetites for what is always escaping." The acutely sick or injured patient's appetite is often poor, and, left to himself, he simply does not eat enough. But diet control alone is inadequate; it must be but a part of the total care of the patient. We do not treat the biochemical data, we treat the patient; the biochemical data are just one factor which helps us determine the status and needs of the patient. Specific antibiotic or chemotherapeutic therapy is clearly of paramount importance in the treatment of infections; appropriate, surgical procedures, skilfully and knowledgeably performed, are essential for the proper care of the injured patient. We must modify, in as favorable a manner as possible, the underlying clinical disorder.

Injury. There is general agreement that no 'strenuous' attempt be made to 'completely' overcome the early increased urinary nitrogen excretion in the first few days following severe injury. The major effort here is the prevention or treatment of shock and maintenance of fluid and electrolyte balance by replacement of lost blood, plasma and electrolytes by appropriate transfusions and infusions; we also give fairly large doses of vitamin C and the B complex. Gastrointestinal function is often impaired at this time, especially if the patient has been in shock; oral feeding, too vigorously forced at this time, may result in acute nausea, vomiting, gastric dilatation or intestinal ileus. Very soon though, one can and should begin and gradually increase a comprehensive dietary regimen. It is our feeling (though we cannot substantiate this with incontrovertible proof) that the negative nitrogen balance should be 'reasonably' minimized early by providing,

fairly rapidly, a full and 'adequate' diet. Oral feedings may occasionally need parenteral supplementation, but in our experience, this is uncommon unless there has been specific damage to the gastrointestinal tract (118). The food provided should be a complete metabolic mixture, containing high quality protein, carbohydrate, minerals, water and accessory food substances. Unfortunately, we do not know what the optimal quantitative relationships of the various metabolites should be.

Severely burned children can be maintained in excellent nutritional condition when they are given dietary intakes 1–1½ times their normal 'requirements' (as outlined by the National Research Council Food and Nutrition Board). Severely burned women have been kept in good nutritional states by supplying them about 45 calories and 2–4 gm of protein/kg body weight. Young adult males on a dietary intake similar to females show greater metabolic disturbances for a comparable injury; it seems that a daily intake of 3–4 gm protein (Bull (119) has recommended up to 6) and 50–60 calories/kg body weight are desirable. If too high a carbohydrate intake is given early, serious hyperglycemia, glycosuria and dehydration may result. This can be alleviated to a large extent if fat makes up from 30 to 40% of the total calories; we have at times gone up to 50% of the calories from fat at the very high caloric levels. If renal failure is present, these types of diets, of course, are not suitable. The problems in the management of such patients has been described by Meroney (120).

The vitamin requirements of the seriously injured are not definitely known. We have been giving five times the normal requirements of the B vitamins, about ten times that of vitamin C, and one or two times the normal requirements of the fat soluble vitamins.

If serious protein depletion has occurred in the severely injured patient, nutritional repletion must be undertaken. The methods and timing of nutrient supply will depend on the clinical situation. Effective treatment depends on recognizing and correcting the underlying cause and providing proper nutrition. If emergency or urgent operative treatment is required, efforts will have to be directed primarily to replacing red blood cells, plasma proteins, water, electrolytes, vitamins and carbohydrate deficits by the parenteral (principally intravenous) route—little can be accomplished in a very short time towards correcting generalized tissue protein deficiencies. If the time of operative intervention is not urgent, significant improvement of the nutrition can be accomplished by a controlled regimen, combining oral, tube and parenteral feedings given gradually and progressively over a period of weeks.

Acute Infectious Diseases. The relation of nutrition to infection is no simple, or single, problem; we have already discussed some aspects of this problem. Schneider (121) commenting on a group of papers presented at a Symposium on Nutrition in Infections in 1955, made the following penetrating statements:

"With few exceptions, the contributors to this monograph have leaned towards the view that in striving for abundance, in the nutritional sense, we are on the side of the angels, and that, when we come to contemplate the consequences for infectious disease of this abundance, only the happy end of increased resistance confronts us. Now, as experimenters in the field have been aware, this just is not so. For example, students of infectious disease have long recognized that in viral disease it is only the well-nourished host which has maximal susceptibility, and it is the malnourished host which has an advanced resistance. . . .

"I think there is one more hidden assumption implicit in many of the papers presented here. This is the silent acceptance of the myth of panresistance, the idea that 'resistance to infection' is a status embracing resistance to *all* infections. That this has been shown to be not only a myth, but an incorrect myth, we owe to the geneticists who have clearly demonstrated, by inbreeding and selection, that it is possible to assemble into one and the same host susceptibility to one disease and resistance to a second. This means that it must be anticipated that in relating nutrition to infection, there must be introduced some motions of specificity; i.e., specific nutrients must be examined in relation to specific infections."

He also pointed out that procedures such as vaccination, chemotherapy and antibiosis are important and effective ways of coping with certain infections and that nutrition 'on the face of things as they now are' falls below these in an hierarchical order.

But the benefits which may arise from appropriate nutritional treatment of the patient with an acute infectious disease are dramatically pointed out by DuBois (2).

"Although typhoid fever may be mild in its course, the majority of cases are severe enough to cause great prostration, and these severe diseases are so greatly influenced by treatment that they may be divided into two groups which differ from each other as much as if they were two different diseases. In the first group we have the

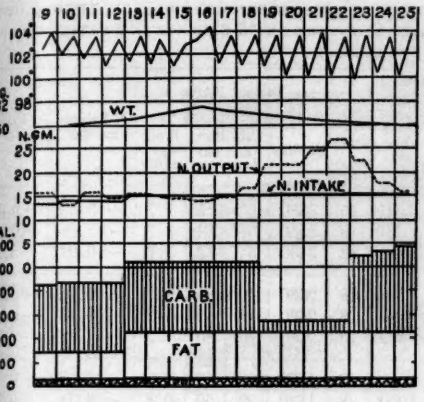


FIG. 3. Typhoid patient Z-O, studied by Shaffer and Coleman. He was maintained in nitrogen equilibrium by giving over 5000 calories per day. The line of dashes represents the urinary nitrogen plus 15% of the food nitrogen, which is a liberal estimate for the amount excreted in the feces. (DuBois, (2).)

patients who are kept on the old fashioned semi-starvation diet of broths, lemonades with egg albumin and milk in limited amounts. These show the classical picture of the so-called 'typhoid state' with emaciation, delirium, tremors, etc. In the second group are the patients who are given liberal diets of more than 2000 calories a day. These show but slight emaciation and suffer so little from nervous symptoms that they read newspapers every day. This is the real picture of typhoid fever; the other is a combination of typhoid and inanition. . . . In typhoid fever the food does not cause any significant rise in energy consumptions. . . . The practical application of this is that we need not be afraid of increasing the fever by giving food."

Shaffer and Coleman (4) were apparently the first to bring typhoid patients into nitrogen balance by giving diets rich in carbohydrate, moderate in protein. But the calories needed were far in excess of that needed to cover the daily expenditure as measured in the calorimeter. "From this it is clear that it requires about 58 to 85 calories per kilogram in the food to bring the patients into nitrogen balance although they produce on an average about 40 calories per kilogram, allowing for the modest amount of muscular work performed by typhoid patients" (DuBois).

In summary, specific antibiotic, chemotherapeutic, antitoxic, etc. therapy are of prime importance in the care of the patient with infectious disease. Appropriate diethotherapy, though, at least for bacterial infections, is a most valuable

TABLE 5. FOOD REQUIRED TO BRING TYPHOID PATIENTS INTO NITROGEN EQUILIBRIUM

Name	Day of Disease	Range of Max. Temp.	Av. Food Cal/Kg/24 Hr.	Av. Food N/24 Hr.	Av. N Balance /24 Hr.
R. N.[1]	20–24	104.4–102.0	71	10.9	+1.5
U. H.[1]	27–30	100.4–101.0	68	8.9	+0.4
Z. O.[1]	9–12	104.0–103.2	72	13.9	−0.2
Z. O.[1]	13–18	103.6–102.8	85	15.0	+0.6
Charles N.[2]	28–31	102.0–101.8	69	18.7	+6.0
Michael K.[2]	15–17	102.0–100.5	79	17.9	+1.5
Philip R.[2]	14–15	102.0–102.0	67	17.6	+2.7
John N.[2]	26–30	102.0–104.0	58	17.8	+1.9
Frank W.[3]	24–28	101.0– 99.6	74	20.3	+4.9
F. Ma.[4]	6–8	103.1–103.6	45.5	10.5	−0.5
J. Gerl[5]	9–10	103.6–103.8	59.5	7.5	+0.4
J. Gerl[5]	16–21	100.9–103.4	76.6	8.1	+0.03

[1] Shaffer and Coleman. [2] DuBois. [3] Coleman and DuBois. [4] Rolland. [5] Rolland: Boy aged 10, weight 26 kg. (DuBois, (2).)

adjuvant; again nutritional prophylaxis and therapy, to be most effective, must be integrated into the total program of patient care.

CHRONIC INFECTIOUS DISEASE

Brief mention only will be made of the possible interrelations between nutrition and chronic bacterial infectious disease. Chronic viral disease will not be included in the discussion. In general the interrelations between nutrition and chronic bacterial infectious disease are of the same complexity as those already mentioned in the preceding section on acute infectious disease.

It has been inferred that subacute bacterial infections generally, including tuberculosis, lead to N wasting and/or depress N storage (122). Statistical evidence relating the prevalence of tuberculosis to poor socio-economic conditions, including poor nutrition, is well-known (10). That anorexia accompanying the disease completes a vicious cycle is also evident (123). McCann and Barr (124) showed that the total energy metabolism in tuberculosis tends to be proportional to the temperature. When they gave patients with tuberculosis diets containing more calories than required to cover their energy expenditure determined by calorimetry but only about 3 gm N daily they were unable to reduce N excretion below 5–6 gm/day. Later, however, McCann (125) reported N retention in tuberculous patients when they received from 70 to 140% more calories than their estimated post-

TABLE 6. AGE, SEX, WEIGHT, DIAGNOSIS, NUTRITIONAL STATUS, PREDNISONE DOSAGE, LENGTH OF OBSERVATION PERIOD, CALORIE AND PROTEIN DIETARY INTAKE AND N BALANCE IN 6 PATIENTS WITH NEOPLASTIC DISEASE

Patient	Age, (in yrs), Sex	Final Control Weight kg	Diagnosis	Nutritional Status	Prednisone Daily Dose mg	No. of Days	Dietary Intake				N Balance gm/day	N Balance gm/kg/day
							Cal/day	Cal/kg/day	Prot/day gm	Prot/day gm/kg		
FB	22, ♂	65.12	Hodgkin's granuloma	Excellent	0	16	3530	54	112	1.7	+ 0.32	+0.005
					100	8	3530	54	112	1.7	− 2.68	−0.041
					1000	12	3530	54	112	1.7	−10.04	−0.154
AR	71, ♂	67.30	Multiple myeloma	Good	0	16	2660	40	80	1.2	+ 0.98	+0.015
					100	8	2660	40	80	1.2	− 6.75	−0.100
					1000	12	2660	40	80	1.2	−11.34	−0.168
WE	67, ♂	62.09	Prostatic carcinoma	Poor	0	12	1820	29	50	0.8	− 2.68	−0.043
					1000	8	1820	29	50	0.8	− 8.74	−0.141
					0	16	1820	29	50	0.8	− 0.58	−0.009
			39-day interval mean dose 85 mg/day									
		54.69			250	12	1800	33	45	0.8	− 6.10	−0.112
WC	60, ♂	60.0*	Chronic lymphocytic leukemia	Fair	0	16	2790	47	96	1.6	+ 2.59	+0.043
					50	16	2790	47	96	1.6	− 2.13	−0.036
					100	8	2790	47	96	1.6	− 3.14	−0.052
VC	65, ♀	51.97	Lymphosarcoma	Fair	0	16	1650	32	51	1.0	− 0.13	−0.002
					100	8	1650	32	51	1.0	− 5.48	−0.105
					1000	8	1650	32	51	1.0	− 9.35	−0.180
					100	8	1650	32	51	1.0	− 3.72	−0.072
					0	16	1650	32	51	1.0	+ 0.56	+0.011
JC	53, ♀	63.75	Multiple myeloma	Good	0	19	1910	30	56	0.9	+ 0.16	+0.003
					100	8	1910	30	56	0.9	− 2.56	−0.040
					1000	8	1910	30	56	0.9	− 5.86	−0.092

* Initial control weight.

FIG. 4. Prednisone and Cremalin (Winthrop-Stearns) dosages, metabolic balances, actual and theoretical weights, serum uric acid concentrations, urinary uric acid excretions and hematologic data of a 22-year-old man with Hodgkin's disease of 15 mo. known duration before, during and after prednisone administration while he was consuming a 3530 calorie diet containing 112 gm protein, 154 gm fat and 424 gm carbohydrate. Cremalin was administered to help protect the patient from peptic ulcer during the administration of the large doses of prednisone. The balance charts in this and subsequent figures are constructed according to the general scheme proposed by Reifenstein *et al.* (145). Intake is plotted downward from the zero line; fecal and urine outputs are then plotted upward from the line representing intake. When total output touches the zero line, equilibrium is represented; when output fails to reach the zero line, positive balance is represented; when output extends beyond the zero line, negative balance is represented. The ordinates of the N, K, P and Ca graphs are so selected that equal heights represent the approximate ratios at which these elements exist in normal protoplasm and bone, i.e. 3 gm of N to 8 mEq K to 200 mg P (protoplasm) and 400 mg Ca to 200 mg P (bone). The ordinates of the Na and Cl graphs are so selected that equal heights represent the approximate ratios at which these elements exist in normal extracellular fluid, i.e., 150 mEq of Na to 100 mEq Cl. Below the bar graphs, line graphs record the patients' weights. In addition to the actual weights, theoretical weights based on considerations of the gain or loss of intracellular and extracellular fluid quantified by balances of N, K and Na are depicted and identified. The line graphs below the weight curves represent the serum uric acid concentration and the bar graphs, the excretions of uric acid. The Arabic numerals along the top and bottom of the chart refer to the metabolic balance periods of a particular study. Except where otherwise indicated, these periods were of 4 days' duration. (Figs. 4–9 from Lund and Levenson in 2nd edition of *Operative Technic, General Surgery*, by W. H. Cole, M.D. New York, Appleton, 1955.)

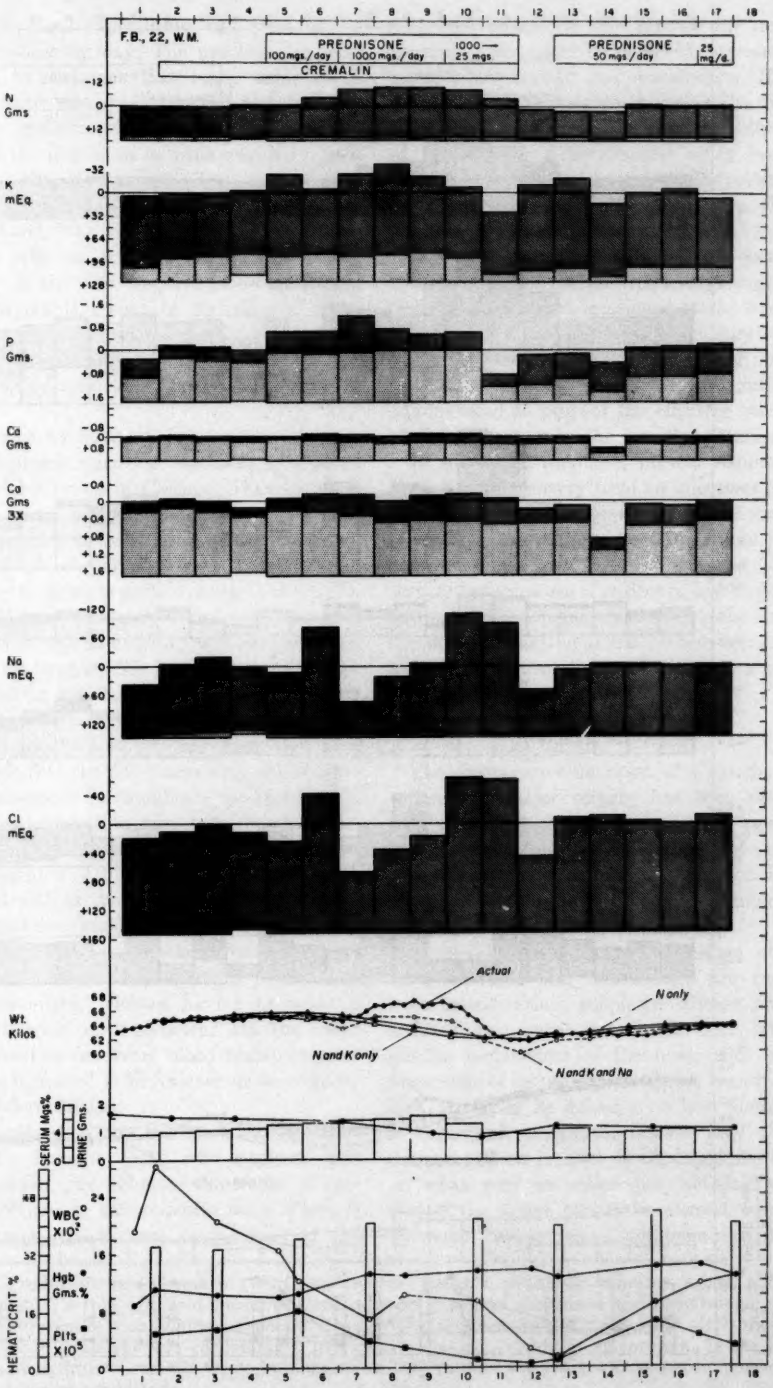

F.B., 22, W.M.
PREDNISONE
100 mgs./day | 1000 mgs./day | 1000 — 25 mgs.
CREMALIN
PREDNISONE
50 mgs./day | 25 mg./d.
N Gms
K mEq.
P Gms.
Ca Gms.
Ca Gms 3X
Na mEq.
Cl mEq.
Wt. Kilos
Actual
N only
N and K only
N and K and Na
SERUM Mgs%
URINE Gms.
HEMATOCRIT %
WBC X10³
Hgb Gms.%
Plts X10⁵

FIG. 4

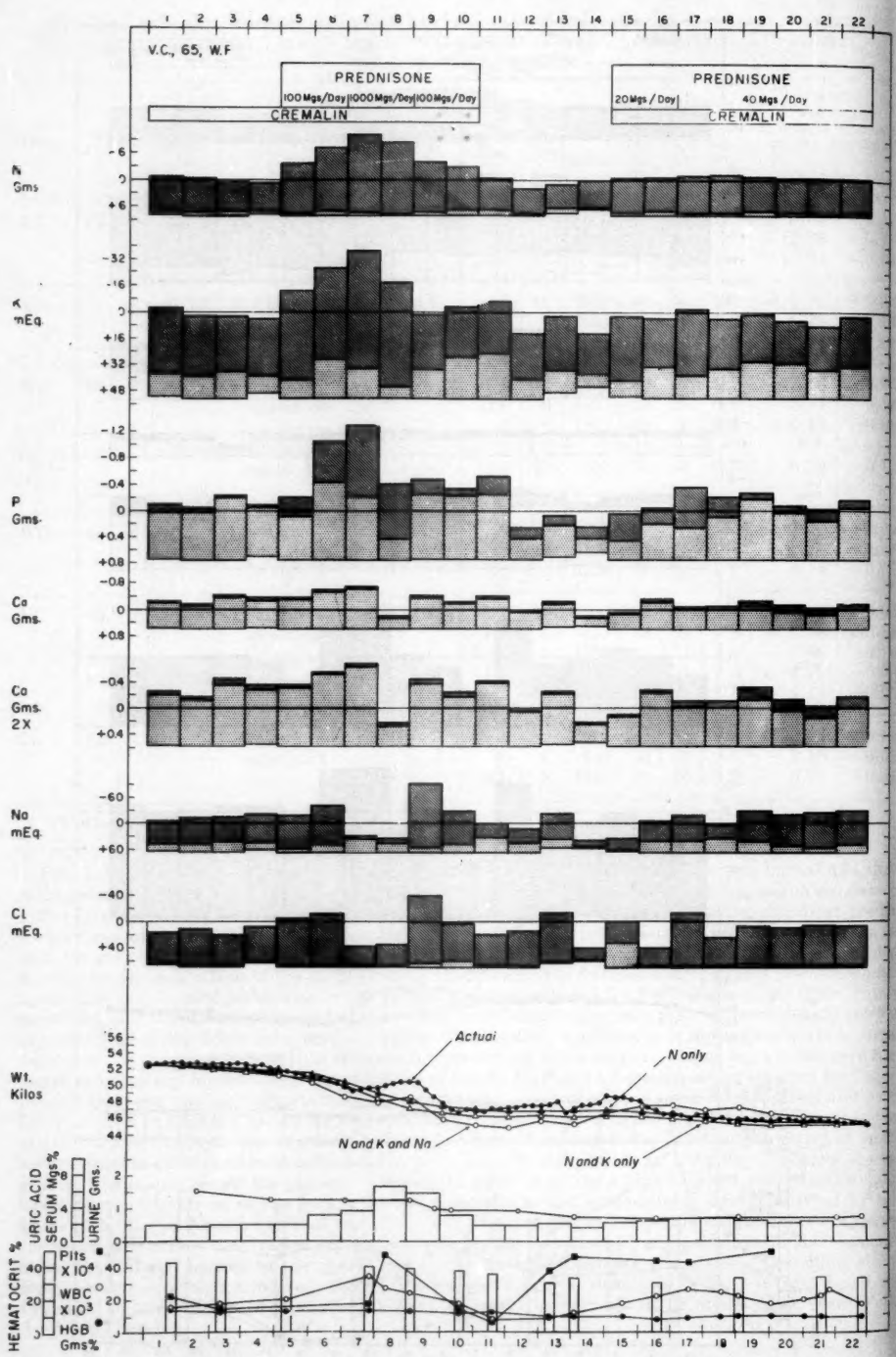

V.C., 65, W.F
PREDNISONE
100 Mgs/Day | 1000 Mgs/Day | 100 Mgs/Day
CREMALIN
PREDNISONE
20 Mgs / Day | 40 Mgs / Day
CREMALIN
N Gms
K mEq.
P Gms.
Ca Gms.
Ca Gms. 2X
Na mEq.
Cl mEq.
Wt. Kilos
Actual
N only
N and K and Na
N and K only
URIC ACID SERUM Mgs%
URINE Gms.
HEMATOCRIT %
Plts X 10⁴
WBC X 10³
HGB Gms%

FIG. 5

absorptive basal expenditure and from 0.7 to 2.9 gm/protein/kg/day. The problem has been confused by the fact that many tuberculosis patients have been studied at bed rest and that the only measure of resistance has been the course of the disease in patients who have been exposed to the infection (10). The overwhelming body of clinical evidence collected, especially during World War II, points to caloric undernutrition with or without qualitative dietary deficiency as the most frequent factor associated with unfavorable course of the disease. Keys (10) has reported relapses among American prisoners of war with tuberculosis who had at first responded remarkably to rest and abundant feeding.

Resistance to infection has specifically been related to protein nutrition, especially by Cannon (126) and his group in Chicago. They have repeatedly shown a diminished agglutination titer under experimental conditions using low protein diets and plasmapheresis after immunization with many antigens in protein deficient rats (127). Metcoff (128) was unable to find a definite relation between protein nutrition and antibody production. Postwar studies in Germany, although demonstrating the superiority of antibody production by well-nourished over undernourished hospital patients and prisoners, led Gell (10) to conclude that the differences were not of practical significance. To complicate the picture still further, undernutrition may provide protection against certain infectious disease (129). It has been suggested (10) that undernutrition is associated with an increased prevalence of infections primarily involving the mucocutaneous barrier for which explanations based on high dosages of infectious material due to poor sanitation are available without having to evoke a general decrease in 'resistance.' On the other hand, infections involving blood transport seem not to be increased in prevalence or severity by severe undernutrition.

One explanation for this paradox has been offered by Kühnau (130) who suggests that protein intake may influence the course of bacterial infections in two opposite ways. First, it may enhance the defense mechanisms of the *host*, and, second, it may provide the invading bacteria with nutritive material necessary for growth, propagation and invasiveness. He suggests that the prevalence of one or the other of these two mechanisms determines whether lack of protein has a detrimental or a favorable effect on the infected organism. He presents an impressive body of evidence indicating the importance of host protein in establishing resistance to bacterial infections. However, he feels that protein requirements are frequently exaggerated and that even losses occasioned by the release of adrenocortical steroids are of short duration. He suggests that the ideal management of infection calls for a high protein intake with antibiotics administered to prevent the effective utilization of dietary protein by the invading organisms.

In summary, therefore, protein requirements for optimum recovery from an infectious process are dependent to a large extent on the nature of the illness and on the factors other than protein nutrition prevalent in the particular circumstance. Judicious use of antibiotic and antipyretic agents is more capable of influencing the status of the disease than the actual protein intake. The nutritional care of the patient is but a part of his comprehensive treatment.

NEOPLASTIC DISEASE

The preoperative nutrition of a patient about to undergo major surgery has been discussed briefly above; these same general principles apply to the preoperative preparation of a patient with neoplastic disease. Our discussion in this section will concern primarily the problems of patients with incurable neoplasms.

If complications such as intestinal obstruction, infection and hemorrhage are excluded from consideration, neoplastic disease produces stress in two ways: first, it exerts an influence on the metabolism of the host; and, second, knowledge of its presence produces anxiety in the host. As far as its influence on host metabolism is concerned, neoplastic disease may produce dramatic effects or none at all, depending largely on what may be called the 'activity' of the disease. In active neoplastic disease, evidenced by weight loss, increased metabolic rate, wasting

FIG. 5. Prednisone and Cremalin (Winthrop-Stearns) dosages, metabolic balances, actual and theoretical weights, serum uric acid concentrations, urinary uric acid excretions and hematologic data of a 65-year-old woman with lymphosarcoma of 2 mo. known duration before, during and after prednisone administration while she was consuming a 1650 calorie diet containing 51 gm protein, 74 gm fat and 194 gm carbohydrate. Cremalin was administered to help protect the patient from peptic ulcer during administration of prednisone.

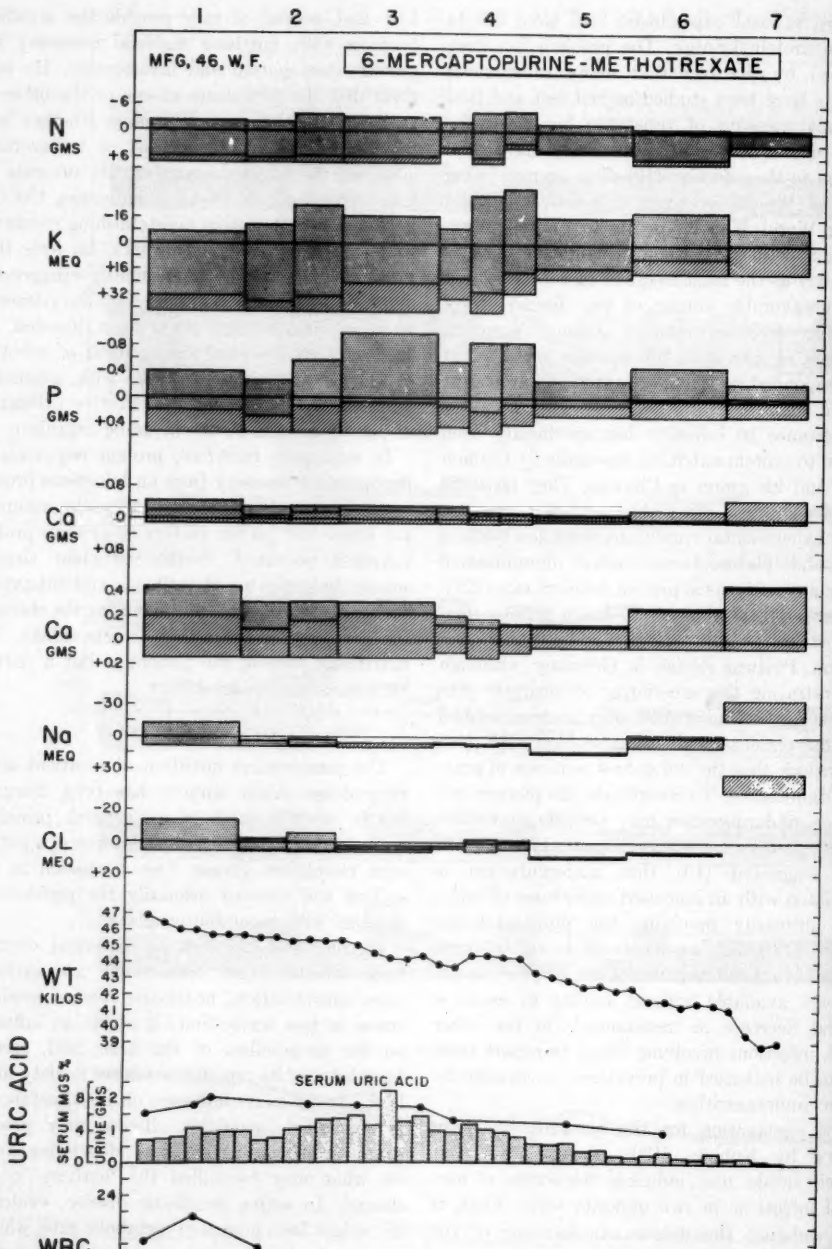

1
2
3
4
5
6
7
MFG, 46, W, F.
6-MERCAPTOPURINE-METHOTREXATE
N GMS
K MEQ
P GMS
Ca GMS
Ca GMS
Na MEQ
CL MEQ
WT KILOS
URIC ACID
SERUM MGS %
URINE GMS
SERUM URIC ACID
WBC X10³
HGB GMS%
TRANF.
TRANF

FIG. 6

of fat stores, elevated serum uric acid concentrations, anorexia, anemia and elevated plasma unesterified fatty acid concentrations, the nitrogen balance may be negative. An example of this is depicted in figure 6, a study of a lady with acute leukemia, and in figure 7, a study of a man with a rapidly growing chondrosarcoma. Despite maximally tolerated orally administered diets and, in the case depicted in figure 7, intravenous caloric supplementation, N storage was not attained. On the other hand, figure 8 depicts positive N balance and weight gain in a patient with chronic myelocytic leukemia after reduction in the activity of his disease by chemotherapy. To complicate the problem still further, some patients with active disease may be in negative calorie balance and yet retain N through changes in body composition (131). Studies of over-all energy expenditures suggest negative calorie balances are characteristic of patients with active, progressive disease (64, 131–136). The interrelation of calorie and protein nutrition raises the question of whether the negative N balances observed in some patients indicate *a*) protein breakdown to provide amino acid building blocks for tumor growth or *b*) protein breakdown to provide energy by way of gluconeogenesis. Moreover, the presence of positive N balances in some patients (131, 136) suggests either the ability of the neoplastic cell to outdo normal tissues in the capture of amino acids with resulting decrease in normal tissue anabolism (137) or the deposition of protein elsewhere than in tumor or muscle (138). The answer to this problem is not simple. First of all, as can be seen in table 7 (JB), the N/P balance ratio in active neoplastic disease is low, implying a loss of a tissue high in P or the selective trapping of N by tumor or the deposition of N elsewhere in the host. The concept of the tumor as a 'nitrogen trap' (139) has appeal and is well-substantiated by animal experimentation. It is, however, hard to reconcile with quantitative data obtained in human studies, since the tumor rarely attains the size necessary to account for the 'missing' N. Liver hypertrophy may provide a more plausible explanation as far as human studies are concerned (138).

The prolonged glucose tolerance curves in patients with cancer (140), the low respiratory quotients found in basal, fasted patients with neoplastic disease (141), the high concentrations of plasma unesterified fatty acids (142) and the changes in body composition characterized by marked fat loss (135, 136) imply that patients with active neoplastic disease may utilize increased amounts of fat and suggest that protein may be burned as fat or ketone bodies after preliminary conversion to carbohydrate or ketone bodies (143). Definitive investigations of this possibility have yet to be taken in man.

To distinguish in quantitative terms between active and inactive neoplastic disease and to appraise the effects of variation in calorie and protein content of the diet, Tschudy *et al.* (144) studied a 50 kg, 54-year-old woman with lymphosarcoma during an inactive phase of her disease on 4 diets providing four combinations of calorie and protein intake and during active and post-radiation phases on the low calorie, low protein diet used in the initial study. (Fig. 9).

Metabolic balances were studied by classical techniques (145), calorie expenditures were measured by the insensible water loss technique of Newburgh *et al.* (146) and the dynamic aspects of N metabolism were estimated by the San Pietro and Rittenberg (147) model for measurement of the nitrogenous metabolic pool size (P), the rate of N incorporation into the protein of the patient (S) and the rate constant for metabolic pool turnover (B).

They observed that a 40 gm/day protein intake during the inactive phase of her disease was associated with weight loss and negative N balance on both 1800 and 1200 calorie diets. An 80 gm/day protein intake was associated with slight N retention but continued weight loss (fig. 9) on the 1200 calorie diet and with slight N retention and weight gain on the 1800 calorie diet. The dynamic studies suggest that the rate constant for metabolic pool turnover (B) is relatively constant and unaffected by changes in calorie and protein intake over the range of dietary variation and period of time studied. The nitrogenous metabolic pool size (P) and turnover rate (BP) appear to increase somewhat when

FIG. 6. Metabolic balances, body weights, serum uric acid concentrations, urinary uric acid excretions and hematologic data before and during chemotherapy with a combination of 6-mercaptopurine and Methotrexate of a 46-year-old woman with acute lymphocytic leukemia previously untreated, of 5 mo. known duration. In this figure the 2nd Ca chart is identical to the 1st except that the scale has been expanded approximately 3 times to provide easier reading. The metabolic periods referred to by the Arabic numerals at the top are of 6 days' duration except for period 7 which is of 5 days'. (Reproduced with permission of the publisher from *Am. J. Med.* 24: 638–647, 1958.)

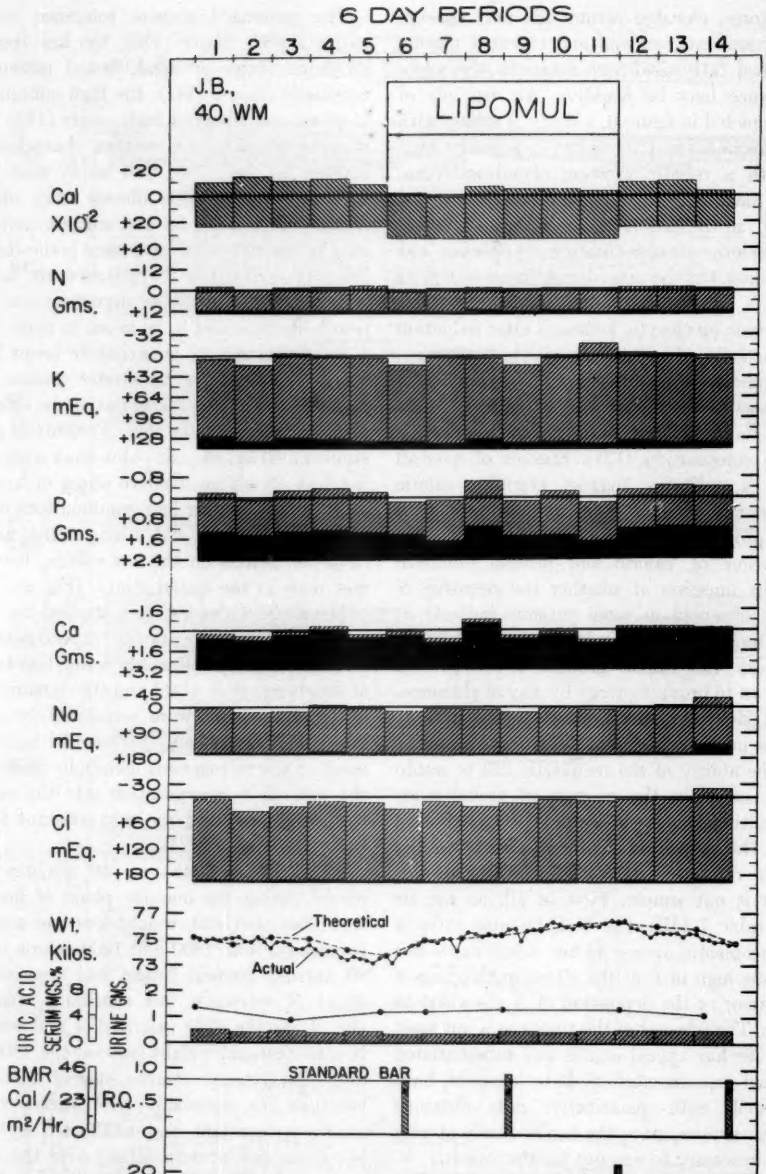

6 DAY PERIODS
1 2 3 4 5 6 7 8 9 10 11 12 13 14
J.B.,
40, WM
LIPOMUL
Cal X10² -20 0 +20 +40
N Gms. -12 0 +12 -32
K mEq. 0 +32 +64 +96 +128
P Gms. -0.8 0 +0.8 +1.6 +2.4
Ca Gms. -1.6 0 +1.6 +3.2
Na mEq. -45 0 +90 +180
Cl mEq. -30 0 +60 +120 +180
Wt. Kilos. 77 75 73
Theoretical
Actual
URIC ACID SERUM MGS.% 8 4 0
URINE GMS. 2 1 0
BMR 46
Cal / 23 m²/Hr. 0
R.Q. 1.0 .5 0
STANDARD BAR
BSP % 20 0
HEMATOCRIT % 50 40 30 20 10 0
Hgb. Gms.% 20 16 12 8 4 0
WBC X10³
Plts. X10⁵
1 2 3 4 5 6 7 8 9 10 11 12 13 14
6 DAY PERIODS

FIG. 7

the dietary protein was increased from 40 to 80 gm/day. The rate of N incorporation into the protein of the patient (S) increased only when both N and caloric intake were raised. Since, on the 80 gm/day protein intake, the total N and urea excretion were essentially the same on both the 1200 and 1800 calorie diet, the greater incorporation into protein (S) corresponds to more rapid turnover of the metabolic pool (BP) associated with the higher caloric intake. Although S increased significantly on the high protein, high calorie regimen, the patient retained an average of only 0.32 gm N/day, not significantly different from the 0.35 gm N/day retained on the high protein low calorie regimen. This suggests that high protein, high calorie diets may increase protein turnover and incorporation when there is no demonstrable net increase in the amount of protein in the body. The patient on leaving the hospital gained 7 kg over the course of the next 6 months during which time her activity, appetite and *ad lib.* food intake were increased. This poses the question as to whether increased protein turnover may be a necessary prelude to positive N balance.

When this patient returned 1 year after discharge with active neoplastic disease and was again studied, this time on a 1200 calorie, 40 gm protein diet (the maximum oral intake she could tolerate), an increase in nitrogenous metabolic pool size (P) but no change in the rate constant for metabolic pool turnover (B) were found. The metabolic pool turnover rate (BP) and the rate of N incorporation into protein (S) were increased as they had been in the preceding study when both calorie and protein intake were at their highest levels.

Shortly after these investigations were completed, the patient was given 1000 roentgens of X-irradiation directed at a growing lymphosarcoma on the leg. Studies performed within 12 hr. revealed a marked reduction in the rate constant for metabolic pool turnover (B) and a marked rise in metabolic pool size (P). The metabolic pool turnover rate remained essentially constant.

The results of the studies performed when the disease was inactive are in general agreement with investigations in animals (148), in normal human subjects (149) and in patients with chronic liver disease (150), suggesting that incorporation of N into protein is greatest when both calorie and protein intakes are high. The results of studies performed when the disease was active suggest that the turnover rate of the nitrogenous metabolic pool (BP) and the N incorporation into protein (S) were equal to those observed during high calorie, high protein feedings. This raises the important question as to whether the consumption of more dietary calories and protein during periods of increased disease activity will further increase protein turnover and incorporation and, if so, whether these increases result in *a*) net N retention or *b*) merely increase energy and protein metabolism. Indirect evidence obtained from balance study data (133, 136) suggest that the latter *b*) may occur. There is also some evidence that such increased feedings may be harmful from the point of view of control of the neoplasm; actuarial statistics (151, 152) and animal experiments (153–155) have emphasized the tumor prevention aspects of caloric restriction in man and the protective effects of caloric restriction in the tumor-bearing animal. In their studies on caloric hyperalimentation, Watkin and Steinfeld (133) and Terepka and Waterhouse (156) observed increased disease activity with provision of more calories accompanied by increased caloric expenditures. In other circumstances, caloric supplementation has resulted in dramatic improvement of the patient (157, 158). The distinction must again be made between those patients with active and inactive disease. The former may be harmed by excessive feeding (159), whereas the latter may be benefited.

The impact of emotional stress in neoplastic disease and its relation to protein metabolism deserves considerable attention. Many patients with relatively inactive or even benign neoplasms will manifest weight loss, high metabolic rates and negative N balances for months because of gnawing fear of the disease. On being reassured, tranquillized if need be, given some

FIG. 7. Metabolic balances, actual and theoretical body weights, serum uric acid concentrations, urinary uric acid excretions, bromsulphalein (BSP) retention and hematologic data of a 40-year-old man with a chondrosarcoma of the lumbar spine of 2.5 yr. known duration before, during and after a 36-day phase of parenteral supplementation with 600 ml daily of a 15 per cent emulsion (Lipomul, I. V., Upjohn) of cottonseed oil added to an orally administered 2400 calorie liquid formula diet containing 88 gm protein, 94 gm fat and 300 gm carbohydrate. The metabolic periods referred to by the Arabic numerals at the top and bottom are of 6 days' duration. (See table 7 also.)

valid advice, they may improve markedly and, aside from periodic check-ups, can be treated as normal subjects. The influence of emotions on nitrogen metabolism has been pointed out by Schottstaedt (160) who observed marked negative changes in N balance induced by stressful situations arising on a metabolic ward. If a change of rooms, a reprimand by a nurse or even anxiety among the nursing staff can sharply increase N loss by the patient, it is not surprising that knowledge that he harbors a cancer may result in chronic N loss. The emotional factors influencing metabolism in human neoplastic disease are so prevalent as to constitute a part of the disease syndrome and perhaps more than other items point out the difference between studying neoplasia in patients and in animals.

In summary, in the psychologically well-adjusted patient with neoplastic disease, N turnover and loss seems in general to be proportional to the activity of the disease, not to the presence of disease. In active neoplastic disease, protein loss and turnover is greater than in normals or in patients with inactive disease. The practical answer does not seem to lie in force feeding of protein and calories or in qualitative changes in protein intake but rather in reversing the trend of the disease. This may often be done temporarily at least by radiation, surgery, or chemotherapy. The beneficial results obtained in this symptomatic or palliative sense provide ample justification for therapy of patients with 'incurable' neoplastic disease.

CHRONIC LIVER DISEASE

The role of protein in the pathogenesis and treatment of clinical Laennec's cirrhosis of the liver has been investigated extensively and many excellent contributions are available (165–167).

Opinion on dietary protein in the management of cirrhosis may for convenience be separated into two major schools, the 'high protein' and the 'adequate protein.' Arguments of certain proponents of the two schools will be presented. Recent developments in the medical management of cirrhosis bearing directly on protein will be examined. Brief mention will be made of the role of dietary protein in the management of patients with viral hepatitis.

Morey *et al.* (168) (representative of the high protein school), collected clinical, metabolic and laboratory data on 4 men with cirrhosis of the liver, each studied for 166 days while each subsisted on 4 different therapeutic regimens. Their plan was to develop a metabolic mixture providing the best environment for maximal growth and reproduction of hepatic parenchymal cells and for repairing secondary disturbances as soon as possible. The patients ranged in age from 32 to 60, were alcoholics, and had experienced such symptoms and signs as anorexia, weight loss, increasing abdominal girth, swelling of legs and ankles, spider angiomata, jaundice, ascites, hematemesis, splenomegaly and hepatomegaly for periods ranging from 8 months to 2 years prior to the beginning of these studies. Two of the patients had been observed previously during metabolic studies on a diet containing 1.0 gm protein/kg body weight and 1.4 times basal calories for periods of 160 and 40 days, respectively.

The authors had not been satisfied with the restoration of depleted tissue in these two patients and in other patients receiving 1.0 gm/kg body weight/day and 1.4 times basal calories, although they agree with the observation of Klatskin and Yesner (163) that this protein intake when accompanied by rest and alcohol withdrawal eventually resulted in clinical improvement. In this study, therefore, they gave the 4 patients a control diet containing 2.5 gm protein/kg body weight, 2 times basal calories (30% fat) for periods of 18 days prior to and following each of 3 other experimental periods. These also were administered for 18 days each; they were: *1*) 120 gm of amino acids (10% I. V. solution of Interchemical Corporation's low sodium lyophilized amino acid product for parenteral use, Elamine) added to the control regimen; *2*) cystine and choline (each 3 gm/day), methionine (5 gm/day), vitamin B complex orally with Intraheptol (liver extract, Lederle) intravenously added to the control regimen; and *3*) human serum albumin (25% solution,

FIG. 8. Colcemide (Ciba) dosage, metabolic balances, calorie balances, actual and theoretical body weights, serum uric acid concentrations, urinary uric acid excretions, basal metabolic rates (BMR) and basal respiratory quotients (RQ) and hematologic data of a 53-year-old man with chronic myelocytic leukemia, previously untreated, of 3 mo. known duration before, during, and after courses of Colcemide while he was consuming orally a 2490 calorie diet containing 85 gm protein, 110 gm fat and 290 gm carbohydrate.

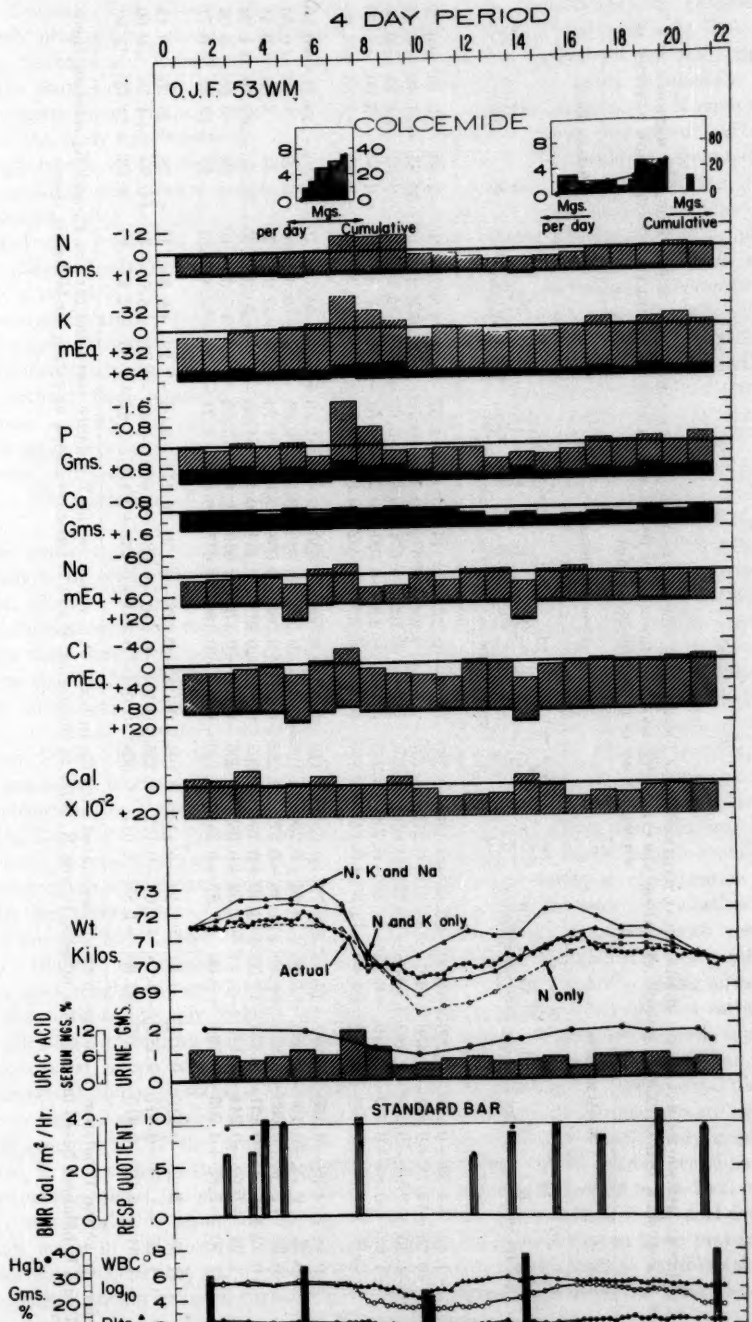

4 DAY PERIOD
O.J.F. 53 WM
COLCEMIDE
Mgs. per day
Mgs. Cumulative
N Gms.
K mEq.
P Gms.
Ca Gms.
Na mEq.
Cl mEq.
Cal. X 10⁻²
N, K and Na
N and K only
Actual
N only
Wt. Kilos.
URIC ACID SERUM MGS. %
URINE GMS.
STANDARD BAR
BMR Cal./m²/Hr.
RESP QUOTIENT
Hgb. Gms. %
Hct %
WBC log10
Plts.* log10
4 DAY PERIOD

FIG. 8

TABLE 7. ACTUAL METABOLIC BALANCES, THEORETICAL BALANCES OF P (CORRECTED FOR CA BALANCE) AND OF K BASED ON N/P RATIOS, K/P RATIOS AND K/N RATIOS FOR MUSCLE AND LEUKEMIC TISSUE AND APPROXIMATE NA/CL RATIO IN NORMAL EXTRACELLULAR FLUID AND BALANCE RATIOS FOR PATIENT JB WHOSE STUDY IS DEPICTED IN FIGURE 7

J.B. Period	Actual Balances						Theoretical Balances							Balance Ratio			
	N gm/day	K mEq/day	P_β gm/day	Ca gm/day	Na mEq/day	Cl mEq/day	$P_\beta = \frac{Ca}{2.23}$ mg/day	$P_{\alpha-\beta} = P_\alpha - \frac{Ca}{2.23}$ gm/day	$P_\mu = \frac{N}{14.7}$ gm/day	$P_\lambda = \frac{N}{7.3}$ gm/day	$P_\mu = \frac{K}{40}$ gm/day	$P_\lambda = \frac{K}{30}$ gm/day	$K_\mu = 2.7N$ mEq/day	$K_\lambda = 4.2\ N$ mEq/day	$Na = 1.5\ Cl$ mEq/day	$\frac{N}{P_{\alpha-\beta}}$	$\frac{K}{N}$	$\frac{K}{P_{\alpha-\beta}}$	$\frac{Na}{Cl}$
1	−1.62	+4.54	−0.200	+0.290	+1.90	+5.85	+0.130	−0.330	−0.110	−0.222	+0.114	+0.151	−4.37	−6.80	+8.78	4.91	−2.80	−13.76	+0.32
2	−0.26	+14.78	+0.008	+0.480	+19.71	+34.81	+0.215	−0.207	−0.018	−0.036	+0.370	+0.493	−0.70	−1.09	+52.22	1.26	−56.85	−71.40	+0.57
3	−1.45	−2.94	−0.258	+0.102	+16.08	+23.72	+0.046	−0.304	−0.099	−0.199	−0.074	−0.098	−3.92	−6.09	+35.58	4.77	+2.03	+5.75	+0.68
4	−1.68	+0.84	−0.372	−0.310	−0.75	+11.56	−0.139	−0.233	−0.114	−0.230	+0.021	+0.280	−4.54	−7.06	+17.34	7.21	−0.50	−3.60	−0.06
5	−2.17	+2.07	−0.214	+0.314	+8.66	+12.80	+0.141	−0.355	−0.148	−0.297	+0.052	+0.069	−5.86	−9.11	+19.20	6.11	−0.95	−5.83	+0.68
6	+0.44	+14.65	+0.156	+0.082	+14.38	+28.73	+0.037	+0.119	+0.030	+0.060	+0.366	+0.488	+1.19	+1.85	+43.10	3.70	+33.30	+123.11	+0.50
7	−0.32	+0.72	+0.106	+0.659	+5.66	+10.60	+0.296	−0.190	−0.022	−0.044	+0.018	+0.024	−0.86	−1.34	+15.90	1.68	−2.25	−3.79	+0.53
8	−0.64	+2.33	−0.660	−0.830	+6.88	+21.96	−0.372	−0.288	−0.044	−0.088	+0.058	+0.078	−1.73	−2.69	+32.94	2.22	−3.64	−8.09	+0.31
9	−0.52	+16.55	+0.131	+0.433	+17.32	+13.49	+0.194	−0.063	−0.035	−0.071	+0.414	+0.552	−1.40	−2.18	+20.24	8.25	−31.83	−262.70	+1.29
10	−1.24	+1.97	−0.244	+0.117	−0.44	+5.02	+0.052	−0.296	−0.084	−0.170	+0.049	+0.066	−3.35	−5.21	+7.53	4.19	−1.59	−6.66	−0.09
11	−1.70	−1.88	+0.205	+0.769	+6.06	+8.57	+0.345	−0.140	−0.082	−0.164	−0.047	−0.063	−3.24	−5.04	+12.86	8.57	+1.57	+13.43	+0.71
12	−1.88	−5.22	−0.310	−0.274	−0.84	+5.50	−0.111	−0.199	−0.128	−0.258	−0.130	−0.174	−5.08	−7.90	+8.25	9.45	+2.78	+26.23	−0.15
13	−1.53	−1.36	−0.447	−0.272	−2.78	+4.64	−0.122	−0.325	−0.104	−0.210	−0.034	−0.045	−4.13	−6.43	+6.96	4.71	+0.89	+4.18	−0.60
14	−1.34	+5.27	−0.415	−0.105	−32.00	−1.88	−0.047	−0.368	−0.091	−0.184	+0.132	+0.176	−3.62	−5.63	−2.82	3.64	−3.93	−14.32	−17.0

P_α refers to actual P balance. P_β refers to the P associated with Ca in bone. $P_{\alpha-\beta}$ refers to the P balance corrected for Ca. P_μ and K_μ refer to theoretical P and K balances based on ratios for muscle, P_λ and K_λ refer to theoretical P and K balances based on ratios for leukemic tissue.

American National Red Cross), 50 gm/day intravenously plus cystine, choline, methionine, B complex vitamins and Intraheptol as above added to the control regimen.

Over-all improvement in the 4 men over the 126 days of the study was impressive. The high protein, high caloric control regimen itself was strikingly beneficial and induced markedly positive N balances. When N intake was increased experimentally to a total of 40 to 50 gm/day by infusion of 120 gm of amino acids daily, a striking percentage of added N was retained despite a marked increase in urinary total and amino N during this phase of the study. The authors report "from a clinical standpoint and as judged by N retention without fluid retention, and serum cholinesterase activity, the greatest improvement in all patients occurred during and shortly after therapy with the control regimen plus intravenous administration of the amino acid mixture."

When the control regimen was supplemented as described above by orally administered cystine, methionine, choline and vitamins and intravenously administered liver extract, N retention was greater than during the control phase but less striking than during the previous phase of parenteral amino acid administration or the subsequent phase of parenteral albumin administration. When albumin 50 gm/day intravenously was added to the control regimen and other supplements, less N appeared in the urine than during the amino acid infusions, reflecting the differences in renal clearance and in rate of metabolism between albumin and amino acids.

Based on their observations of these 4 patients and of 2 of the same and of other similar patients previously studied on intakes of 1.0 gm protein/kg body weight/day and 1.4 times basal calories, the authors strongly believe in the merits of the 2.5 gm protein/kg body weight/day and 2 times basal calorie regimen. As for the more controversial supplementation of an already high protein regimen with amino acids, they circumspectly comment "If the amino acid is sodium-free, if it does not disturb the appetite or produce nausea, and if the patient can afford it, then . . . it is a valuable adjunct to therapy."

Although many of the authors' conclusions may be warranted by the data and by their clinical impressions, it should be noted that, in order to explain the huge positive N balances and the relatively small gains in weight, they must postulate without supporting evidence "that the muscles of the patients initially contained large quantities of fluid and relatively little protoplasm and that throughout the study this fluid was supplanted by newly synthesized muscle protein." In these patients, the N equivalent of 15–30 kg of new muscle protoplasm was retained over and above what could be accounted for by changes in weight. In addition, it should be observed that the amino acids were infused in all 4 patients during the second 18-day phase of the study. Since the control diet itself was admirable from a nutritional standpoint, it is perhaps unreasonable to conclude that the amino acid infusions, coming as they did early in the total study, had any special advantage of the other regimens. Inspection of the graphs depicting the study further suggests that, in the presence of adequate to superior oral protein and calorie intake, supplementation is certainly not essential; we feel that parenteral amino acid supplementation would only rarely be indicated. By their cautious use of conditional phrases, the authors impart the suggestion at least that they may be in accord with this observation.

One of the chief objections to very high protein intakes by patients with cirrhosis revolves around the problem of ammonia intoxication. The evidence is clear that ammonia arises in the gastrointestinal tract as a result of enzymic hydrolysis of urea by the gut bacteria; if liver function is impaired, the ammonia reaches the hepatic veins and later the systemic circulation in abnormal quantities, ammonia toxicity may result, and, in part, lead to hepatic coma (169).

Plough *et al.* (150), in consideration of this, designed studies to determine whether a large positive N balance in patients with severe liver disease could be maintained at lower, safer levels of dietary protein intake by taking advantage of the protein sparing effect of extra carbohydrate and fat calories. They gave a caloric supplement of 700–1000 calories daily of fat and carbohydrate to patients on experimental diets, providing from 25 to 50 calories/kg body weight and from 1.0 to 4.0 gm protein/kg body weight. They observed that the N stored per supplemental calorie was directly related to the level of protein in the diet. Their studies of skin fold thicknesses led them to suggest that at lower protein intakes in excess of calories leads to the formation of fat, but at higher levels of protein intake leads to the formation of lean tissue.

Gabuzda and Davidson (170) and Davidson (166, 171) (representative of the adequate pro-

tein school) have emphasized that the patient with cirrhosis is best managed on a diet providing 1.0 gm protein/kg body weight and between 1800 to 2200 calories/day for the hospitalized patient. They warn against the dangers of inducing hepatic coma by feeding patients with cirrhosis more than this amount of protein. Davidson (171) states: "There is little evidence that more protein than this will increase the rate of healing or of regeneration of the liver nor does it seem to speed up improvement in nutritional rehabilitation."

In evaluating the arguments of the high protein versus the adequate protein schools of thought, it should be pointed out that variations in patient material and in experimental design may account for some of the differences in viewpoint expressed. Given the same patients at the same stage of the disease, it is possible that most authors would agree on approximately the same dietary regimen. The more acute the illness and the more active the disease, the greater the dangers of inducing ammonia toxicity by protein feeding. The more quiescent the disease, the more beneficial increased protein intake may become. Sborov (165), who himself approves 120–550 gm protein and 3500 calories/day in the chronic stage of the disease, has expressed this sentiment succinctly, stating: "Protein should be given cautiously but, if tolerated, its ingestion should be encouraged."

The problem of hepatic coma has led in recent years to various medical measures based on concepts arising from basic biochemical research designed to carry a patient with hepatic disease through the dangerous phase of the illness characterized by encephalopathy. Three measures will be briefly discussed: the use of glutamic acid and its sodium salt, the use of Krebs-Henseleit urea cycle intermediates and the use of orally administered antibiotics.

Walshe (172) observed the return to consciousness of 3 patients with subacute and chronic liver disease in 5 episodes of hepatic coma shortly after their receiving intravenously or orally 23 gm sodium glutamate. His therapy was suggested by the observations that glutamic acid is one of the few free amino acids occurring in appreciable concentration in brain and is the only amino acid oxidized in brain. It combines with ammonia to form glutamine which is readily excreted without liberating ammonia. Since then the literature has contained many conflicting reports on the usefulness of sodium glutamate in the management of hepatic coma and led McDermott *et al.* (173) to an independent evaluation of 28 patients. They divided these patients into 3 groups: *1*) The 'acute spontaneous encephalopathy' (patients with advanced liver disease who developed encephalopathy without any known precipitating factor); *2*) the 'acute exogenous encephalopathy' (patients with well-compensated livers who had acute episodes of encephalopathy precipitated by the introduction into the gastrointestinal tract of large amounts of such nitrogenous substances as blood, ammonium chloride or high protein feedings); and *3*) the 'chronic encephalopathy' (patients with hepatic or portal disease manifesting chronic confusion without rapid progression to coma and without spontaneous remissions and exacerbations). In the 'exogenous' and 'chronic' groups, ammonia toxicity was apparent and 25 gm daily of *l*-glutamic acid or its sodium salt seemed an effective part of therapy. In the 'acute spontaneous' group, however, whose disordered ammonia metabolism was but one biochemical defect out of many induced by the disease, only transient, if any, improvement was noted; the level of blood ammonia could be reduced but the coma was not corrected. These authors emphasize that glutamate is effective in modifying ammonia metabolism but that this must not be regarded as the only biochemical defect in the disease.

The use of Krebs-Henseleit urea cycle intermediates (*l*-arginine, *l*-citrulline, *d,l*-ornithine) in protecting against amino acid and ammonia toxicity has been reported on numerous occasions (174–180). Addition of the Krebs-Henseleit intermediates may, by increasing the amount of substrate, increase the reaction velocity of the cycle and hence increase the removal of ammonia (181). As in the case of glutamate, the use of Krebs-Henseleit cycle

FIG. 9. Metabolic balances of calories, N, K, P, Ca, Na and Cl; body weight and theoretical weight curves, serum uric acid concentrations and urinary uric acid excretions; and basal metabolic rates (BMR) and respiratory quotients (RQ) of a 54-year-old woman with clinically inactive lymphosarcoma previously treated with x-irradiation of 3.5 yr. known duration studied for 84 days on 1800 calorie, 40 gm protein; 1200 calorie, 40 gm protein; 1200 calorie, 80 gm protein; and 1800 calorie, 80 gm protein diets. (Reproduced with permission of the publisher from *J. Clin. Investigation* 38: 892–901, 1959.)

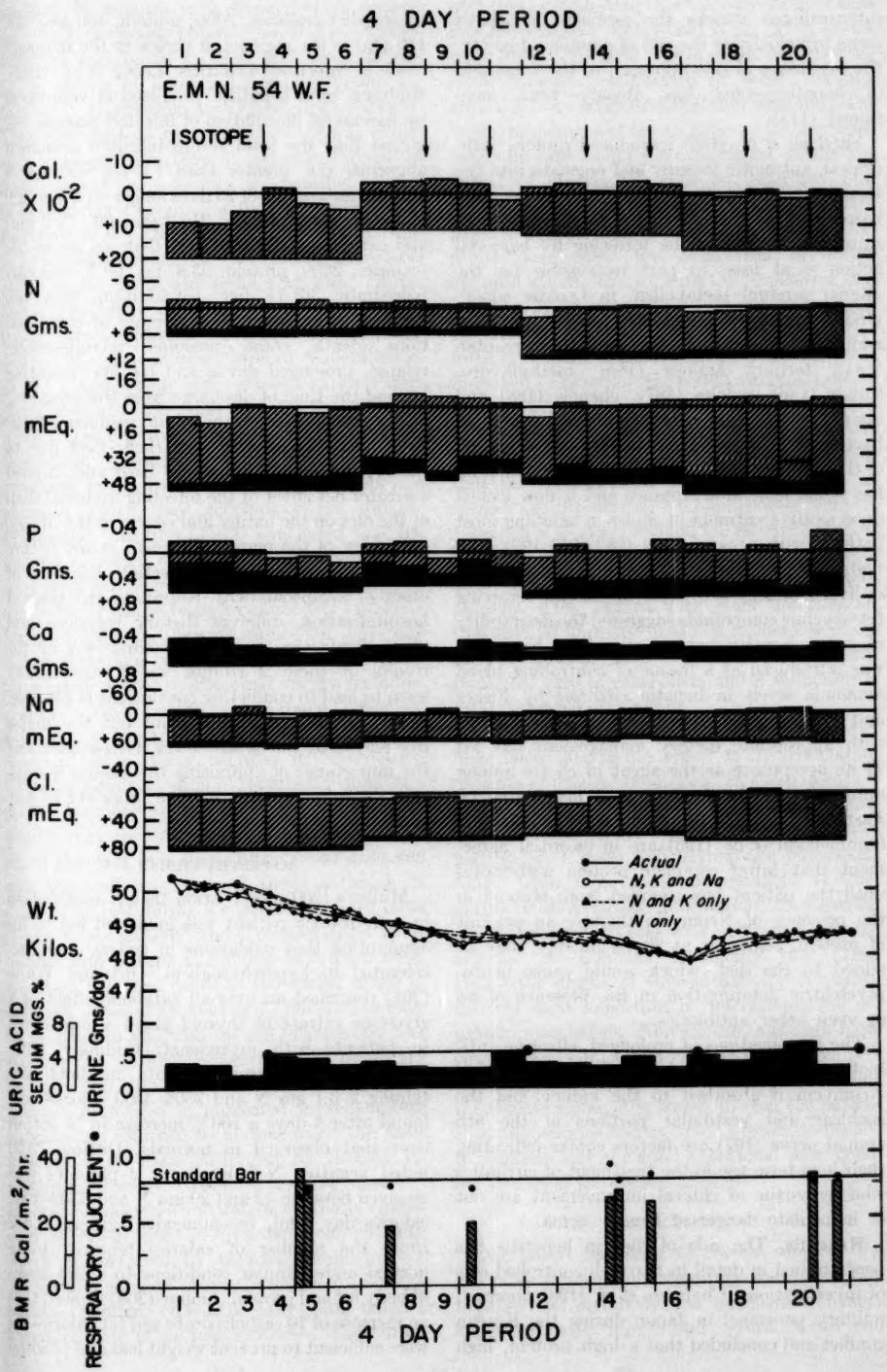

4 DAY PERIOD
E.M.N. 54 W.F.
ISOTOPE
Cal. X 10²
N Gms.
K mEq.
P Gms.
Ca Gms.
Na mEq.
Cl. mEq.
Actual
N, K and Na
N and K only
N only
Wt. Kilos.
URIC ACID SERUM MGS.%
URINE Gms/day
BMR Cal/m.²/hr.
RESPIRATORY QUOTIENT
Standard Bar
4 DAY PERIOD

FIG. 9

intermediates attacks the problem of hepatic coma principally at the site of ammonia toxicity; the limitation of this approach in the treatment of hepatic coma has already been mentioned (173).

Sherlock *et al.* (182) introduced protein withdrawal, antibiotic therapy and enemata into the treatment of hepatic coma. The regimen was based on "the concept that a toxic nitrogenous substance formed in the intestine by bacterial action is at least in part responsible for the altered cerebral metabolism in hepatic coma" (183). Among the suggested toxic agents in addition to ammonia are methyl mercaptan (184), tertiary amines (185), methylhydrosulfide (186), indoles (187), phenols (188) and amines (189). Antibiotics modify the intestinal bacteria and, thereby, may modify the formation of these 'toxic' metabolites. This form of therapy has found general acceptance and is now looked upon as the treatment of choice in handling most patients with hepatic coma (183, 190–193). The finding of nitrogen catabolism and abnormalities in liver histology (194, 195) in patients receiving tetracycline compounds suggested the desirability of using nonabsorbable antibiotics. Neomycin was introduced as a means of controlling blood ammonia levels in hepatic cirrhosis by Fisher and Faloon (190). Its efficacy when combined with appropriate dietary management has led to its acceptance as the agent of choice among antibiotics in the treatment of hepatic coma. Fast *et al.* (191), Stormont *et al.* (192) and Summerskill *et al.* (193) are in essential agreement that (after complete protein withdrawal until the patient has emerged from stupor) in the presence of Neomycin therapy an amount of protein, beginning at 30–40 gm/day, may be added to the diet, which would cause neuropsychiatric deterioration in the presence of no or even other antibiotics.

The complications of prolonged, effective antibiotic therapy (196) and the possible toxicity of Neomycin if absorbed to the kidney and the cochlear and vestibular portions of the 8th cranial nerve (197) are factors contra-indicating their long-term use in the treatment of cirrhotics who by virtue of clinical improvement are out of immediate danger of hepatic coma.

Hepatitis. The role of diet in hepatitis has been studied in detail in two well-controlled sets of investigations. Chalmers *et al.* (198) observed military personnel in Japan during the Korean conflict and concluded that a high protein, high calorie diet (calories, 3000; protein, 150 gm; fat, 150 gm) is the regimen of choice in the management of infectious hepatitis. Leone *et al.* (199), studying 'viral hepatitis' produced in volunteers by parenteral inoculation of infected plasma, observed that the total serum bilirubin remained abnormal (i.e. greater than 1.0 mg/100 ml of plasma) an average of 23 days longer on a 'special' (calories, 3844; protein, 215.2 gm; fat, 79.2 gm; and carbohydrate, 579.4 gm) than on an *ad lib.* (calories, 2609; protein, 93.8; fat, 90.5; and carbohydrate, 361.7) diet. In addition, they observed a more frequent occurrence of complications (death, coma, precoma, recrudescence, relapse, prolonged illness and residua persisting beyond the time of discharge from the program) on the special than on the *ad lib.* regimen. Statistical evaluation showed a marked effect due to the type of infecting material used and implied a greater net effect of the infecting material than of the diet on the nature and course of the illness.

In view of the many differences in the nature of the virus, the mode of infection, the time of onset of symptoms with respect to the time of hospitalization, different dietary regimens and other conditions, no direct comparison of the results of these 2 studies which superficially seem to lead to conflicting conclusions is possible. They do, however, again point out the major role played by the nature of the disease itself and the importance of appraising the disease quantitatively before considering the propriety of any given dietary regimen.

HYPERTHYROIDISM

Müller's (200) observation that N metabolism in a thyrotoxic patient was increased led to his assumption that oxidations in general were accelerated in hyperthyroidism. Ord and White (201) described an over-all catabolic effect of a glycerine extract of thyroid gland administered to patients with myxedema. Rudinger (202) placed 2 hyperthyroid patients on diets containing 2–3.1 gm N and 2500–3500 calories and found after 4 days a 100% increase in N output over that observed in normals. DuBois (203) noted negative N balances in 2 patients who received between 12 and 20 gm N and 3500–4000 calories/day and recommended giving 1.5–2 times the number of calories required by a normal under similar conditions to avoid losses of body fat and protein. Janney (204) showed that an increase of 10 carbohydrate and fat calories/kg were sufficient to prevent weight loss and produce

positive N balance in a hyperthyroid patient who had previously been studied by DuBois. Boothby and Sandiford (205, 206) gave calories far in excess of basal requirements before attaining N equilibrium in hyperthyroid patients. They showed that part of this excess was necessary because hyperthyroid patients expend at least twice as many calories as do normal persons in performing a given amount of muscular work. Boothby *et al.* (207) produced N equilibrium in hyperthyroid patients with diets of 3000–5000 calories and 1–1.5 gm protein/kg/day. Lauter and Jenke (208) and Krauss (209) found that hyperthyroid patients on minimal N intakes reached the same minimal level of N metabolism as did normals provided sufficient calories were given. Boothby (quoted in Lusk (210)) attributed much of the negative N imbalance seen in hyperthyroidism to such complications as gastrointestinal crises, nausea, vomiting, diarrhea and anorexia which may accompany the disease. Peters and Van Slyke (211) concluded that the thyroid secretion causes no specific destruction of protein but merely increases the demands of the body for fuel. Except for cautiously excluding low protein diets, they recommended only high calorie diets and rest as a means of preventing the wastage caused by abnormal energy demands.

Janney (204) noted an anabolic action of thyroxine in therapeutic doses (0.75 mg/day) in a cretin but a 'toxic' decrease in N retention following administration of far smaller quantities of the hormone to euthyroid patients ill with non thyroid diseases. Deuel (212) gave himself 7 mg of thyroxin in a single intravenous dose and was observed to excrete 3 times as much N daily as he had during a control period. In more recent investigations where combinations of N balance and isotope techniques have been used, attention has been called to the varied effects of different doses of thyroid and *l*-triiodothyronine on N balance and also on the degradation and synthesis of protein. Schwartz (213) found the biologic half life of human iodinated serum albumin prolonged in untreated myxedematous patients and returned to normal when the same patients were made euthyroid by thyroid replacement therapy. Crispell *et al.* (214) observed an over-all decrease in protein synthesis (measured by N^{15}-glycine) in myxedematous patients, which is corrected by thyroid administration. They administered *l*-triiodothyronine to a healthy human volunteer (100 μgm daily), and noted a decreased rate of protein synthesis. They interpret this as suggesting that the *l*-triiodothyronine was antianabolic. Rothschild *et al.* (215), giving large doses (720–1080 mg) of desiccated thyroid, noted increased catabolism of serum albumin and a fall in total exchangeable albumin. The latter, however, was not so great as the increased catabolic rate would have produced during the period of observation, and so suggested that synthesis was also increased. Iber *et al.* (216) present evidence to suggest that *l*-triiodothyronine accelerates the catabolism of serum albumin simultaneously with the production of a negative N balance. They concluded that accelerated catabolism was the cause of the negative nitrogen balance and that albumin synthesis was increased to maintain the serum albumin concentration. They also suggest from data collected from euthyroid subjects on isocaloric high protein (3.0 gm/kg) and low protein (0.5 gm/kg) diets that the catabolism of serum albumin was increased significantly on the high protein regimen. Lewallen *et al.* (217) studied the effects of thyroid hormone on the distribution kinetics, synthesis and degradation of iodoalbumin in myxedematous patients. They agreed with other investigators that thyroid hormone produces an increase in the rate of albumin synthesis, an increase in the fractional rate of catabolism and a reduction in total exchangeable albumin.

Unfortunately these studies of protein kinetics have been carried out either in myxedematous patients or euthyroid subjects. In those instances where exogenous thyroid or *l*-triiodothyronine was administered, the doses used were sufficiently high to place them well beyond the physiologic range. That large doses are catabolic is compatible with available information; that smaller doses given to subjects with subnormal thyroid activity are anabolic is equally true (218). Hence the degree of thyroid activity is quantitatively most important in determining the net protein metabolism. Nonetheless, although more efficient and clinically advisable to improve protein nutrition in hyperthyroidism by controlling the disease alone, it is wisest to feed the patient as many calories and as much protein as needed to maintain him in a good nutritional state pending control of the disease (219).

CONCLUSION

Our intent has been to review some of the available information regarding the connection between the metabolic and nutritional changes associated with injury and certain acute and

chronic diseases and recovery therefrom. The complexity of the problem has been described, the need for concrete, objective study has been indicated. When we consider the difficulties in establishing optimal nutritional requirements for healthy people, it is not surprising that no firm, final recommendations of the protein requirements of the sick and injured can be made now. The approach and needs will vary depending on the individual patient, the nature of his underlying injury or illness, and the urgency of the situation, e.g. the need for operative intervention. It should be emphasized again that the nutritional care of the patient is but a part of his total care; we must modify, in as favorable a manner as possible, his underlying clinical disorders. Our aim is to return the patient effectively to his accustomed place in society as soon as possible.

"It is difficult, seeing that there is no such accuracy in the Art, to hit always upon what is most expedient, and yet many cases occur in medicine which would require this accuracy, as we shall explain. But on that account, I say, we ought not to reject the ancient Art, as if it were not, and had not been properly founded, because it did not attain accuracy in all things, but rather, since it is capable of reaching to the greatest exactitude by reasoning, to receive it and admire its discoveries, made from a state of great ignorance, and as having been well and properly made, and not from chance." (1)

REFERENCES

1. The Genuine Works of Hippocrates (trans. from the Greek by Francis Adams). Baltimore: Williams & Wilkins, 1939.
2. DuBois, E. F. Metabolism in Fever and in Certain Infections. Endocrinology and Metabolism, Vol. 4. L. F. Barker, Ed., New York: Appleton, 922.
3. Müller, F. *Centralbl. klin. Med.* 1884, 5, 569. Quoted by G. Lusk, *The Elements of the Science of Nutrition*, 4th ed. Philadelphia: Saunders, 1928.
4. Shaffer, P. A. and W. Coleman. *A. M. A. Arch. Int. Med.* 4: 538, 1909.
5. Coleman, W. and E. F. DuBois. *A. M. A. Arch. Int. Med.* 15: 887, 1915.
6. Cuthbertson, D. P. *Brit. M. Bull.* 10: 33, 1954.
7. Moore, F. D. and M. R. Ball. *The Metabolic Response to Surgery*, Springfield, Ill., Thomas, 1952.

8a. Selye, H. *Stress*. Montreal: Acta Inc., 1950, 1951.

8b. Selye, H. and A. Horava. *Stress*. Montreal: Acta Inc., 1952, 1953.

8c. Selye, H. and G. Heuser. *Stress*. New York: MD Pub'ns., 1954, 1955/56.

9. *Transactions of Conferences on Bone and Wound Healing and on Metabolic Aspects of Convalescence* with Supplementary Reports (meetings 1–17). New York: Macy, 1942–1951.
10. Keys, A., J. Brozek, A. Henschel, O. Mickelsen and H. L. Taylor. *The Biology of Human Starvation*. Minneapolis: Univ. of Minnesota Press, 1950.
11. Levenson, S. M. *Nutritional and Metabolic Requirements in Relation to Radiation Injuries and Thermal Burns*. Prepared for Subcommittee on Radiation and Thermal Burns, National Research Council Committee on Therapeutic Nutrition, Food and Nutrition Board, 1950.
12. Wyse, D. M. and C. J. Pattee. *The Metabolic Alterations of Immobilization, Injury and Paraplegia*. D. V. A. Treatment Services Bulletin, parts I–V, 1953.
13. Cannon, P. R. *Mil. Med.* 117: 222, 1955.
14. Braasch, J. W. *Surg. Gynec. & Obst.* 88: 473, 1949.
15. Rhoads, J. *Surg. Gynec. & Obst.* 94: 417, 1952.
16. Peters, J. P. Ann. New York Acad. Sc. xlvii (Art. 3): 327, 1946.
17. Elman, R. *Surgical Care; A Practical Physiologic Guide*. New York: Appleton, 1951.
18. Hardy, J. D. *Surgery and the Endocrine System*. Philadelphia, Saunders, 1952.
19. Benedict, F. G. *A Study of Prolonged Fasting*. Washington, D. C.: Carnegie Institute of Washington, Publ. No. 203, 1915.
20. Cannon, W. B. *Bodily Changes in Pain, Hunger, Fear, and Rage*. New York: D. Appleton & Co., 1925.
21. Lusk, G. *The Elements of the Science of Nutrition*, 4th ed. Philadelphia: Saunders, 1928.
22. Moore, F. D. Ann. New York Acad. Sc., 73, Art. 2: 387, 1958.
23. Moore, F. D., J. L. Langohr, M. Ingebretsen and O. Cope. *Ann. Surg.* 132: 1, 1950.
24. Hirshfeld, J. W., H. H. Williams, W. E. Abbott, C. G. Heller and M. A. Pilling. *Surgery* 15: 766, 1950.
25. Meroney, W. H. and R. F. Herndon. *J. A. M. A.* 155: 877, 1954.
26. Levenson, S. M., J. M. Howard and H. Rosen. Surg. Gynec. & Obst. 101: 35, 1955.
27. van Bekkum, D. W. and R. A. Peters. *Quart. J. Exper. Physiol.* 36: 127, 1951.
28. Taylor, F. H. L., S. M. Levenson, C. S. Davidson, N. C. Browder and C. C. Lund. *Ann. Surg.* 118: 215, 1943.
29. Tagnon, H. J., S. M. Levenson, C. S. Davidson and F. H. L. Taylor. *Am. J. M. Sc.* 211: 88, 1946.
30. Zamecnik, P. C., M. L. Stephenson and O. Cope. *J. Biol. Chem.* 158: 135, 1945.
31. Beloff, A. and R. A. Peters. *J. Physiol.* 103: 461, 1945.
32. Rawlinson, W. A. and C. H. Kellaway. *Australian J. Exper. Biol. & M. Sc.* 22: 69, 1944.
33. Kellaway, C. H. and W. A. Rawlinson. *Australian J. Exper. Biol. & M. Sc.* 22: 83, 1944.
34. Clark, E. J. and R. J. Rossiter. *Quart. J. Exper. Physiol.* 32: 279, 1944.

35. GREEN, H. N. AND H. B. STONER. *Biological Actions of the Adenine Nucleotides*. London: Lewis, 1950.
36. GREEN, H. N. AND H. B. STONER. *Brit. M. Bull.* 10: 38, 1954.
37. SCOTT, R., JR., J. M. OLNEY AND J. M. HOWARD. *Battle Casualties in Korea*, vol. I. Washington, D. C.: Walter Reed Army Institute of Research, 1956, p. 149.
38. POLLACK, H. AND S. L. HALPERN. *Therapeutic Nutrition*. Washington, D. C.: National Academy Science-Nutritional Research Council, 1952.
39. MOORE, F. D. *Ann. Surg.* 137: 289, 1953.
40. BUTTERFIELD, W. J. H. *Lancet*, I: 489, 1955.
41. TAYLOR, F. H. L., S. M. LEVENSON AND M. A. ADAMS. *New England J. Med.* 231: 437, 1944.
42. CUTHBERTSON, D. P. *Quart. J. Med.* 25: 233, 1932.
43. COPE, O., G. L. NARDI, M. QUIJANO, R. L. ROVIT, J. B. STANBURY AND A. WIGHT. *Ann. Surg.* 137: 165, 1953.
44. MUUS, J. AND E. HARDENBERGH. *J. Biol. Chem.* 152: 1, 1944.
45. CAIRNIE, A. B., R. M. CAMPBELL, J. D. PULLOR AND D. P. CUTHBERTSON. *Brit. J. Exp. Path.* 38: 504, 1957.
46. MOORE, F. D. AND I. S. EDELMAN. *Surg. Gynec. & Obst.* 95: 155, 1952.
47. WILKINSON, A. W. *Body Fluids in Surgery*. Edinburgh: Livingstone, 1955.
48. CUTHBERTSON, D. P. *Brit. J. Surg.* 23: 505, 1936.
49. HOWARD, J. E., W. PARSON AND R. S. BINGHAM, JR. *Bull. Johns Hopkins Hosp.* 77: 291, 1945.
50. HOWARD, J. E., W. PARSON, K. E. STEIN, H. EISENBERG AND V. RERDT. *Bull. Johns Hopkins Hosp.* 75: 156, 1944.
51. BROWNE, J. S. L., V. SCHENKER AND J. A. F. STEVENSON. *J. Clin. Invest.* 23: 932, 1944.
52. CUTHBERTSON, D. P. *Biochem. J.* 24: 1244, 1930.
53. CUTHBERTSON, D. P. *Biochem. J.* 23: 1328, 1929.
54. MADDEN, S. C. In: *Plasma Proteins*, edited by J. B. YOUMANS. Springfield, Ill., Thomas, 1950, p. 62.
55. LEVENSON, S. M., J. W. BRAASCH, H. MUELLER AND L. CROWLEY. *Nitrogen Metabolism Following Thermal Injury*. (In preparation.)
56. BLOCKER, T. P., JR., W. C. LEVIN, W. W. NORVINSKI, S. R. LEWIS AND V. BLOCKER. *Ann. Surg.* 141: 589, 1955.
57. STERLING, K., S. R. LIPSKY AND L. J. FREEDMAN. *Metabolism* 4: 343, 1955.
58. PFLÜGER, E. *Pflüger's Arch. ges. Physiol.* Bd. XVIII, pp. 303, 356, 1878. Quoted by G. LUSK, *The Elements of the Science of Nutrition*, 4th ed. Philadelphia: Saunders, 1928.
59. LINSER, P. AND J. SCHMID. *Deutsches Arch. klin. Med.* 79: 514, 1904.
60. VON LEYDEN AND KLEMPERER. VON LEYDEN'S *Handbuch der Ernährungstherapie*, Bd. I,I p. 345, 1904. Quoted by G. LUSK. *The Elements of the Science of Nutrition*, 4th ed. Philadelphia: Saunders, 1928.
61. KOCHER, R. A. *Deutsches Arch. klin. Med.* 115: 82, 1914.
62. GROSSMAN, C. M., T. S. SAPPINGTON, V. A. BURROWS, P. H. LAVIETES AND J. P. PETERS. *J. Clin. Invest.* 24: 523, 1945.
63. HOWARD, J. M. (ed.) *Battle Casualties in Korea:* vol. I, Washington, D. C.: Walter Reed Army Medical Center, 1954.
64. WATKIN, D. M. AND J. L. STEINFELD. *Proc. Am. Assoc. Cancer Research* 2: 258, 1957.
65. BROWNE, J. S. L. *Second Conference on Bone and Wound Healing*. New York.
66. HOWARD, J. E., R. S. BINGHAM, JR. AND R. E. MASON. *Trans. Assoc. Am. Physicians* 59: 242, 1946; *Bull. Johns Hopkins Hosp.* 78: 283, 1946.
67. MUNROE, H. N. AND M. I. CHALMERS. *Brit. J. Exper. Path.* 26: 396, 1945.
68. CANNON, P. R. *Recent Advances in Nutrition with Particular Reference to Protein Metabolism*. Lawrence: Kansas Univ. Press, 1950. (No. 14 of the Porter Lecture Series.)
69. WHIPPLE, G. H. AND S. C. MADDEN. *Medicine* 23: 215, 1944.
70. GOLDENBERG, I. S., L. LUTWAK, P. J. ROSENBAUM AND M. A. HAYES. *Surg. Gynec. & Obst.* 102, #2: 129, 1956.
71. SCHWARTZ, A. E. AND K. E. ROBERTS. *Surgery* 42: 814, 1957.
72. SELLERS, E. A., S. S. YOU AND R. W. YOU. *Endocrinology* 47: 148, 1950.
73. VON EULER, U. S. In: *Stress*. edited by H. SELYE AND G. HEUSER. New York: MD Pub'ns, 1955/56, p. 125.
74. HARTMAN, F. W., W. G. ROSE AND E. P. SMITH. *Am. J. Physiol.* 78: 47, 1926.
75. SELYE, H. *The physiology and Pathology of Exposure to Stress*. Montreal: Acta Inc., Medical Publishers, 1950.
76. HUME, D. M., D. H. NELSON AND D. W. MILLER. *Ann. Surg.* 143: 316, 1956.
77. HARDY, J. D. *Ann. New York Acad. Sc.* 73: 401, 1958.
78. HUME, D. M. AND D. H. NELSON. *Fed. Proc.* 13: 73, 1954.
79. NELSON, D. H. In *Stress*, edited by H. SELYE AND G. HEUSER. New York: MD Pub'ns. 1955/56, p. 169.
80. SELYE, H. *The Story of the Adaptation Syndrome*. Montreal: Acta, Inc., Medical Publishers, 1952.
81. HARRIS, G. W. *Neural Control of the Pituitary Gland*. London: Arnold, 1955.
82. HUME, D. *Ann. Surg.* 138: 548, 1953.
83. PORTER, R. W. *Am. J. Physiol.* 172: 515, 1953.
84. ENGEL, F. L. *Endocrinology*, 50: 462, 1952.
85. CAMPBELL, R. M., G. SHARPE, A. W. BOYNE AND D. P. CUTHBERTSON. *Brit. J. Exper. Path.* 35: 566, 1954.
86. INGLE, D. J., E. O. WARD AND M. H. KUIZEGA. *Am. J. Physiol.* 149: 510, 1947.
87. INGLE, D. J. *J. Clin. Endocrinol.* 14: 1272, 1954; In: *Stress*, edited by H. SELYE AND G.

HEUSER. New York: MD Pub'ns. 1955/56, p. 161.
88. SELYE, H.: *J. Clin. Endocrinol.* 14: 122, 1954. H. SELYE AND G. HEUSER (eds.). In: *Stress*, New York: MD Pub'ns. 1955/1956.
89. STEENBURG, R. W. AND W. F. GANONG. *Surgery* 38: 92, 1955.
90. STEENBURG, R. W., R. LENNIHAN AND F. D. MOORE. *Ann. Surg.* 143: 180, 1956.
91. MIRSKY, I. A., M. STEIN AND G. PAULISCH. *Endocrinology* 55: 28, 1954.
92. DEITRICK, J. E. *Bull. New York Acad. Med.* 24: 364, 1948.
93. VAIL, E. *The Influence of Exercise on Nitrogen Metabolism Following Severe Trauma in Adult Male Patients.* Master's Thesis at Medical College of Virginia, Richmond, Virginia, 1952.
94. CROFT, P. B. AND R. A. PETERS. *Nature (London)* 155: 175, 1945.
95. SELLERS, E. A. AND C. H. BEST. *Brit. Med. J.* 1: 522, 1947.
96. PETERS, J. P. *Am. J. Med.* 5: 100, 1948.
97. HOLDEN, W. D., H. KRIEGER, S. LEVEY AND W. E. ABBOTT. *Ann. Surg.* 146: 563, 1957.
98. ABBOTT, W. E., H. KRIEGER AND S. LEVEY. *Bull. Soc. Internat. Chirurg.* 17: 330, 1958.
99. WILKINSON, A. W. *Lancet* II: 428, 1956.
100. WILKINSON, A. W., B. H. BILLING, G. NAGY AND C. P. STEWART. *Lancet* I: 533, 1950.
101. WERNER, S. C., D. V. HABIF, H. T. RANDALL AND J. S. LOCKWOOD. *Ann. Surg.* 130: 688, 1949.
102. LEVENSON, S. M., O. J. MALM, B. W. HAYNES AND E. A. LOUNDS. (In preparation).
103. LUND, C. C. AND S. M. LEVENSON. In: *Operative Surgery.* COLE, W. H. (ed.) New York: Appleton, 1955.
104. RIEGEL, C., C. E. KOOP, R. P. GRIGGER, J. E. RHOADS AND L. BULLITT. *Surg. Clin. North Am.* 25: 1096, 1945.
105. LEVENSON, S. M., C. L. PIRANI, J. W. BRAASCH AND D. F. WATERMAN. *Surg. Gynec. & Obst.* 99: 74, 1954.
106. LEVENSON, S. M., H. L. UPJOHN, J. A. PRESTON AND A. STEER. *Ann. Surg.* 146: 357, 1957.
107. CHASSIN, J. L., H. A. MCDOUGALL, M. MACKAY AND S. A. LOCALIO. *Proc. Soc. Exper. Biol. & Med.* 83, #4: 798, 1953.
108. GLINOS, A. D. *A Symposium on The Chemical Basis of Development*, edited by W. D. MCELROY AND B. GLASS. Baltimore: Johns Hopkins Press, 1958, p. 813.
109. LEVENSON, S. M., L. V. CROWLEY, J. F. OATES AND A. D. GLINOS. *Surg. Forum* 9: 493, 1958.
110. FRANK, E., J. FINE AND L. PILLEMER. *Proc. Soc. Exper. Biol. & Med.* 89: No. 2: 223, 1955.
111. SANFORD, J. P. AND J. R. EVANS. *J. Appl. Physiol.* 10: 88, 1957.
112. DUBOS, R. J. *J. Exptl. Med.* 101: 59, 1955.
113. DUBOS, R. J. AND R. W. SCHOEDLER. *Proc. 4th Internat. Cong. Nutr.* Paris: 1957.
114. DUBOS, R. J. *Lancet* 2: 1, 1955.
115. SMITH, J. M. AND R. J. DUBOS. *J. Exptl. Med.* 103: 109, 1956.
116. BALCH, H. H., JR. *Recent Advances in Medicine and Surgery.* (Medical Science Publication No. 4) Washington, D. C.: Walter Reed Army Medical Center, 1956.
117. GALEN *Galen's Hygiene* (translated by R. M. GREEN), Springfield, Ill.: Thomas, 1951, p. 7.
118. LOUNDS, E. A., R. M. FRANK, M. M. ROBINSON AND S. M. LEVENSON. *J. Am. Dietet. Assoc.* 33: 902, 1957.
119. BULL, J. P. *Proc. Nutrition Soc.* 17: 114, 1958.
120. TESCHAN, P. E. *et al. Am. J. Med.* XVIII, #2: 172, 1955.
121. SCHNEIDER, H. A. *Ann. New York Acad. Sc.* 63, Art. 2: 314, 1955.
122. JOHNSTON, J. A. AND J. W. MARONEY. *J. Pediat.* 12: 563, 1938.
123. DUBOIS, E. F. *Endocrinology and Metabolism.* L. F. BARKER, editor. New York: D. Appleton & Co., 1922, vol. 4, pp. 135.
124. MCCANN, W. S. AND D. P. BARR. *Arch. Internal Med.* 26: 663, 1920.
125. MCCANN, W. S. *Arch. Internal Med.* 29: 33, 1922.
126. CANNON, P. R. *J. A. M. A.* 128: 360, 1945.
127. CANNON, P. R., W. E. CHASE AND R. W. WISSLER. *J. Immunol.* 47: 133, 1943.
128. METCOFF, J., D. DARLING, M. H. SCANLON AND F. J. STARE. *J. Lab. & Clin. Med.* 33: 47, 1948.
129. MARKOWSKI, B. *Brit. Med. J.* 2: 361, 1945.
130. KÜHNAU, J. *Ann. New York Acad. Sc.* 63: 220, 1955.
131. WATKIN, D. M. *Ann. New York Acad. Sc.* 69: 902, 1958.
132. FENNINGER, L. D., C. WATERHOUSE AND E. H. KENTMANN. *Cancer* 6: 930, 1953.
133. WATKIN, D. M. AND J. L. STEINFELD. *Fed. Proc.* 16: 343, 1957.
134. WATKIN, D. M. *Clinical Research* 6: 198, 1958.
135. WATKIN, D. M. *Increased Fat Utilization in the Hypermetabolism of Active Neoplastic Disease.* Abstracts of Papers, Seventh International Cancer Congress, London, July 6–12, 1958, p. 113.
136. WATKIN, D. M. *Acta Unio internat. contra cancrum.* In press.
137. WISEMAN, G. AND F. N. SHADIALLY. *Brit. Med. J.* 2: 18, 1958.
138. WATKIN, D. M. AND R. T. SILVER. *Am. J. Med.* 24: 638, 1958.
139. MIDER, G. B., H. TESLUK AND J. J. MORTON. *Acta Unio internat. contra cancrum* 6: 409, 1948.
140. MARKS, P. A. AND J. S. BISHOP. *J. Clin. Investigation* 36: 254, 1957.
141. WATKIN, D. M. *J. Clin. Investigation* 36: 934, 1957.
142. MUELLER, D. S. AND D. M. WATKIN. *Circulation* 18: 506, 1958.
143. PETERS, J. P. AND D. VAN SLYKE. *Quantitative Clinical Chemistry.* Baltimore: Williams & Wilkins, 1946, p. 22.
144. TSCHUDY, D. P., H. BACCHUS, S. WEISSMAN, D. M. WATKIN, M. EUBANKS AND J. WHITE. *J. Clin. Investigation* (In press).

145. Reifenstein, E. C., F. Albright and S. L. Wells. *J. Clin. Endocrinol.* 5: 367, 1945.
146. Newburgh, L. H., M. W. Johnston, F. H. Loshmet and J. M. Sheldon. *J. Nutrition* 13: 203, 1937.
147. San Pietro, A. and D. Rittenberg. *J. Biol. Chem.* 201: 457, 1953.
148. Allison, J. B. *Ann. New York Acad. Sc.* 69: 1009, 1958.
149. Hegsted, D. M., A. G. Tsongas, D. B. Abbott and F. J. Stare. *J. Lab. & Clin. Med.* 31: 261, 1946.
150. Plough, I. C., F. C. Iber, M. E. Shipman and T. C. Chalmers. *Am. J. Clin. Nutrition* 4: 224, 1956.
151. Fenninger, L. D. and G. B. Mider. *Cancer Research* 2: 229, 1954.
152. Keys, A., J. Brozek, A. Henschel, O. Mickelsen and H. L. Taylor. *The Biology of Human Starvation*, vol. II. Minneapolis: University of Minnesota Press, 1950, pp. 1051–1056.
153. McKay, C. M., G. H. Ellis, L. L. Barnes, C. A. H. Smith and G. Sperling. *J. Nutrition* 18: 15, 1939.
154. Tannenbaum, A. *Am. J. Cancer* 38: 335, 1940.
155. White, F. R., J. White, G. B. Mider, M. G. Kelly and W. E. Heston. *J. Nat. Cancer Inst.* 5: 43, 1944.
156. Terepka, A. R. and C. Waterhouse. *Am. J. Med.* 20: 225, 1956.
157. Pareira, M. D., E. J. Conrad, W. Hicks and R. Elman. *J. A. M. A.* 156: 810, 1954.
158. Pareira, M. D., E. J. Conrad, W. Hicks and R. Elman. *Cancer* 8: 803, 1955.
159. Watkin, D. M. *Proc. Surg. Gen. Task Force on Intravenous Fat Alimentation*. Nashville: Vanderbilt Univ. School of Medicine, 1958, pp. 45–49.
160. Schottstaedt, W. W., R. H. Pinsky, D. Mackler and S. Wolf. *Am. J. Med.* 25: 248, 1958.
161. Patek, A. J., Jr., J. Post, O. D. Ratnoff, H. Mankin and R. W. Hillman. *J. A. M. A.* 138: 543, 1948.
162. Patek, A. J., Jr., and J. Post. *J. Clin. Investigation* 20: 481, 1941.
163. Klatskin, G. and R. Yesner. *J. Clin. Investigation* 28: 723, 1949.
164. Schwarz, K. (editor (consulting) and conf. Chairman) *Ann. New York Acad. Sc.* 57: 615, 1954.
165. Sborov, V. M. *Am. J. Digest. Dis.* 3: 94, 1958.
166. Davidson, C. S. *Am. J. Med.* 25: 690, 1958.
167. Sherlock, S. *Diseases of the Liver and Biliary System*, 2nd ed. Oxford: Blockwell Scientific Publications, 1958.
168. Morey, G. R., C. R. Paynter, C. F. Consolazio and R. M. Kark. *Metabolism* 1: 314, 1952.
169. Phillips, G. B., R. Schwartz, G. J. Gabuzda, Jr. and C. S. Davidson. *New England J. Med.* 247: 239, 1952.
170. Gabuzda, G. J. and C. S. Davidson. *Ann. New York Acad. Sc.* 57: 776, 1954.
171. Davidson, C. S. In: *The Medical Clinics of North America*. Philadelphia: Saunders, 1956, pp. 432–435.
172. Walshe, J. M. *Lancet* 1: 1075, 1953.
173. McDermott, W. V., J. Wareham and A. G. Riddle. *New England J. Med.* 253: 1093, 1955.
174. Gullino, P., M. Winitz, S. N. Birnbaum, M. C. Otey, J. Cornfield and J. P. Greenstein. *Arch. Biochem. & Biophys.* 58: 255, 1955.
175. Najarian, J. S. and H. A. Harper. *Proc. Soc. Exper. Biol. & Med.* 92: 560, 1956.
176. Najarian, J. S. and H. A. Harper. *Am. J. Med.* 21: 832, 1956.
177. Fahey, J. L. *J. Clin. Invest.* 36: 1647, 1957.
178. Bessman, S. P., S. Shear and J. Fitzgerald. *New England J. Med.* 256: 941, 1957.
179. McDermott, W. V., Jr., D. H. Henneman and C. Laumont. *J. Clin. Investigation* 36: 913, 1957.
180. Wolfe, S. J., B. B. Fast, J. M. Stormont and C. S. Davidson. *J. Lab. & Clin. Med.* 51: 672, 1958.
181. Manning, R. T. *Lancet* 1: 937, 1957.
182. Sherlock, S., W. H. J. Summerskill, L. P. White and E. A. Phear. *Lancet* 2: 453, 1954.
183. Sherlock, S., W. H. J. Summerskill and A. M. Dawson. *Lancet* 2: 689, 1956.
184. Challenger, F. and J. M. Walshe. *Biochem. J.* 59: 372, 1955.
185. Butt, H. R. and H. L. Mason. *Gastroenterology* 26: 829, 1954.
186. Phear, E. A. and B. Ruebner. *Brit. J. Exper. Path.* 37: 253, 1956.
187. Walshe, J. M. *Postgrad. M. J.* 32: 467, 1956.
188. Rogers, W. F., M. D. Burdick and G. R. Burnett. *J. Lab. & Clin. Med.* 45: 87, 1955.
189. Melnykowycz, J. and K. R. Johansson. *J. Exper. Med.* 101: 507, 1955.
190. Fisher, C. J. and W. W. Faloon. *New England J. Med.* 256: 1030, 1957.
191. Fast, B. B., S. J. Wolfe, J. M. Stormont and C. S. Davidson. *Arch. Internal Med.* 101: 467, 1958.
192. Stormont, J. M., J. E. Mackie and C. S. Davidson. *New England J. Med.* 259: 1145, 1958.
193. Summerskill, W. H. J. *Brit. Med. J.* 2: 1322, 1958.
194. Faloon, W. W., J. J. Downs and K. Duggan. *Am. J. Med. Sc.* 233: 563, 1957.
195. Faloon, W. W., J. W. Noll and K. Collins. *J. Lab. & Clin. Med.* 44: 75, 1954.
196. Gabuzda, G. J., T. M. Jocke, G. G. Jackson, M. E. Grigsby, B. D. Love, Jr. and M. Finland. *Arch. Internal Med.* 101: 476, 1958.
197. Beckman, H. *Drugs. Their Nature, Action and Use*. Philadelphia: Saunders, 1958, p. 516.
198. Chalmers, T. C., R. P. Eckhardt, W. E. Reynolds, J. G. Cigorroa, N. Deane, R. W. Reifenstein, C. W. Smith and C. S. Davidson: *J. Clin. Invest.* 32: 559, 1952.

199. Leone, N. C., F. Ratner, W. C. L. Diefenbach, M. G. Eads, J. E. Lieberman and R. Murray. *Ann. New York Acad. Sc.*, 57: 948, 1954.
200. Müller, F. *D. Arch. Klin. Med.* 51: 335, 1893.
201. Ord, W. M. and E. White. *Brit. Med. J.* 2: 217, 1893.
202. Rudinger, K. *Wien. klin. Wchnschr.* 22: 1582, 1908.
203. DuBois, E. F. *Arch. Internal Med.* 17: 915, 1916.
204. Janney, N. W. *Arch. Internal Med.* 22: 187, 1918.
205. Boothby, W. M. and I. Sandiford. *J.A.M.A.* 81: 795, 1923.
206. Boothby, W. M. and I. Sandiford. *Physiol. Rev.* 4: 69, 1924.
207. Boothby, W. M., I. Sandiford, K. Sandiford and J. Slosse. *Ergebn. Physiol.* 24: 728, 1925.
208. Lauter, S. and M. Jenke. *Deutsches. Arch. klin. Med.* 146: 323, 1925.
209. Krauss, E. *Deutsches Arch. klin. Med.* 150: 13, 1926.
210. Boothby, W. M. In: *The Elements of the Science of Nutrition*, edited by G. Lusk. Philadelphia: Saunders, 1928, p. 601.
211. Peters, J. P. and D. D. Van Slyke. *Quantitative Clinical Chemistry*, Baltimore: Williams & Wilkins, 1946, p. 668.
212. Sandiford, I., K. Sandiford, H. J. Deuel, Jr. and W. M. Boothby. Quoted by G. Lusk, *The Elements of the Science of Nutrition*, 4th ed., Philadelphia: Saunders, pp. 359–360.
213. Schwartz, E. *J. Lab. Clin. Med.* 45: 340, 1955.
214. Crispell, K. R., W. Parson and G. Hollifield. *J. Clin. Invest.* 35: 164, 1956.
215. Rothschild, M. A., A. Bauman, R. S. Yalow and S. A. Berson. *J. Clin. Investigation* 36: 422, 1957.
216. Iber, F. L., K. Nassau, I. C. Plough, F. M. Berger, W. H. Meroney and K. Freemont-Smith. *J. Clin. Investigation* 37: 1442, 1958.
217. Lewallen, C. G., J. E. Rall and M. Berman. *J. Clin. Investigation* 38: 88, 1959.
218. Paschkis, K. E., A. E. Rakoff and A. Cantorow. Clinical Endocrinology, 2nd Ed. New York: Hoeber, 1958, p. 128.
219. Werner, S. (editor). *The Thyroid, a Fundamental and Clinical Text.* New York: Hoeber, 1955.

PROTEIN, CALORIES AND LIFE EXPECTANCY

Morris H. Ross

Biochemical Research Foundation, Newark, Delaware

Of the numerous factors that have been recognized as playing a role in the states of health and disease and eventually in the death of a living system, nutrition and metabolic patterns attract attention. Metabolic studies have been made of the various types of nutritional deficiencies resulting in disease; however, long-term investigations of the nutritional aspects of metabolism and thereby of degenerative diseases and of life span are, with certain exceptions, notably sparse. On the other hand, the literature is abundant with short-term studies assessing dietary needs, using such parameters as maximum growth rates, specific physiological activities, and utilization or retention of specific dietary constituents; but the effects of life-long consumption of diets which are designed specifically to assess the value of their components are unknown.

Any study of the nutritional effects upon life span will necessitate a comprehensive and chronological analysis of multiple factors. The effects of many intrinsic and interrelated factors such as genetic determinants (1, 2), structural deviations (3), and hormonal balance (4, 5, 6) etc., upon the patterns of early development, upon the processes of growth, maintenance, repair and reproduction and upon disease and senescence, must be recognized as being highly influential in the events leading to the termination of life. Even though such variables may cloud relationships of cause and effect, attempts can be made, nevertheless, to study the effects of extrinsic or environmental factors, e.g. nutrition, upon the condition and length of life. This type of study must encompass total life span in order to reveal the influence of earlier nutritional and metabolic levels upon later metabolic activities, accidents and patterns.

Actuarial data strongly suggest that obesity shortens life span, perhaps through its associated effects on the cardiovascular and renal systems (7, 8). Vital statistics, while perhaps inadequate, do show the existence of a high coefficient of correlation between caloric intake and increase in deaths in man due to arteriosclerotic heart

disease (9–11). Walker (12) has indicated that coronary heart disease is uncommon in nutritionally underprivileged communities. Unfortunately, however, other than for biochemical anthropological studies of isolated human populations (12), and for actuarial information, the elaboration of nutritional effects on organic disease and on life span must be sought within the confines of the laboratory.

At the present time, information on the experimental modification of the duration of life and of degenerative disease processes by overnutrition or oversupplementation, or by undernutrition, is largely incidental to studies germane to gerontological problems.

The consequences of experimental overnutrition on life span are particularly well-defined in the work of Lane and Dickie (13) and of the Silberbergs (14, 15). Lane's results left no doubt that overeating shortened the life of a strain of mice with a genetic proclivity for obesity, and that long-term restriction of food intake would increase the life span of these mice even more than that found for the nonobese controls maintained on the same diet, *ad libitum*. Silberberg and Silberberg (16) reported that the feeding of high fat diets shortened the life span of males of two strains of mice and that life span was shortened more in the longer-lived strain. Similarly, French *et al.* (17), found that rats maintained *ad libitum* on diets high in fat content were shorter-lived than those maintained on the carbohydrate-enriched diet, even though the total caloric intake of the former was less.

Overnutrition in the rat induced by surgical obliteration of centers in the hypothalamus (18) with consequent obesity often resulted in the development of premature renal lesions identical to those found spontaneously in old animals. Goldblatt (19) observed that the changes in renal tissue were the same in old age as those found in obese younger organisms. Similarly, Waxler's (20) studies of mice treated with gold thioglucose to induce overnutrition and consequent obesity revealed a significantly early appearance of mammary tumors. Furthermore, pair feeding to the nonobese untreated mice reduced the incidence in the restricted but treated group to that found in the control group. Overnutrition of rats by forced feeding produced hypothyroidism, possibly as a result of hypofunction of the anterior pituitary (21).

Coronary arterial atheromatous lesions and unusually high blood levels of cholesterol were found in rats maintained on high protein diets (22). The detrimental effect of excess amounts of some amino acids on growth was demonstrated by Russell and his colleagues (23), and Korenchevsky (24) has reviewed the literature on the physiological activity and toxic effects of amino acid oversupplementation.

The effects of undernutrition on life span in various species of invertebrates has been amply reviewed by Lansing (25). Essentially, chronic reduction in food intake or the intermittent feeding of diets apparently 'adequate' in quality retarded growth, delayed development and prolonged the duration of life. For the higher forms of life, various authors have made correlations between length of life and maximum body weight, development of specific structures, physiological functions and nutritional limitations. Carlson and Hoelzel (26, 27) obtained appreciable extension of the life span of rats fed intermittently on diets from animal and vegetable sources, but no significant difference in maximum weights was found.

In well-known studies designed to determine the effects of growth retardation upon life span, McCay and his associates (28) found that there was a definite relationship between retarded growth and length of life. Limiting protein intake with resulting growth retardation was found to double the life span of trout (29). In part these observations support the conclusions of Slonaker (30) that the longest-lived rats in his colony were those that received a meat-containing diet approximately 14% protein. These rats, partially restricted in protein, showed considerably more spontaneous activity and were longer-lived than those of McCay's colony.

In studies on growth retardation of the rat due to caloric restriction, McCay *et al.* (31) used preassigned growth levels during chronological aging as the basis of his feeding program. At given time periods during their lives, these rats were realimented by permitting them to eat the basal ration *ad libitum*. Life spans under these conditions were increased depending on the length of time growth retardation was maintained. It was also found by McCay that retardation of growth for periods up to 700 days did not affect the capacity for further growth in these rats when they were realimented.

Others have reported different results, however, when the ability of rats to recover from the effects of undernutrition was studied. Relatively short-term undernutrition was shown not to affect subsequent growth (32–36). Quimby (37)

even found growth stimulated on realimentation but others have found that retardation of growth during nursing or during early post-weaning periods have a marked dwarfing effect later in life (33, 35, 36, 38).

While confirmation of the effects of caloric restriction, with resultant growth retardation, on life span is seen in the reports of Riesen *et al.* (39), Tannenbaum (40), Rusch *et al.* (41), Carlson and Hoelzel (26), Lee *et al.* (42), the dependence of life span upon growth restriction and delayed maturation has been questioned (17, 43).

The effects of undernutrition are not limited to alteration of life span but are of significant interest as they relate to the state of health, since there are sufficient indications that chronic, age-associated diseases and abnormalities of metabolism do have a nutritional basis. Severe undernutrition of protein, kwashiorkor, is a serious, chronic and widespread problem in human nutrition, and its etiology and treatment have been described by György (44), whereas the anemias and leucopenias (45) have a more immediate relation in time to protein undernutrition.

In the rat, pulmonary disease is reported endemic and the major cause of death (46), but respiratory ailments were markedly reduced in the growth-retarded rats of McCay (47) and of Saxton and Kimball (48). In a similar manner, other age-associated diseases were found to be affected by growth retardation due to caloric restriction (48). Chronic nephrosis, which is a common disease in old, well-fed rats (49), was found to be practically nonexistent in growth-retarded rats. The incidence of periarteritis of the mesenteric arteries (46) observed to be associated with kidney disturbances, particularly in those rats on diets containing high protein supplements, was also reduced in rats whose growth had been retarded due to caloric restriction (48). Though it has been stated that lowered incidence of pulmonary and of degenerative diseases accounts for increase in average life span (50), Riesen *et al.* (39), on the other hand, confirming the effects of calorie restriction on life span, did not find any significant difference in incidences of respiratory ailments.

Statistical records for human beings show a strikingly salutary effect of reduced food intake, particularly caloric, on mortality from cardiovascular affections (51, 52).

A most remarkable delay in time of appearance of and a reduction of the incidence of neoplasia has been found in animals on restricted caloric intake. These include skin tumors (41), mammary tumors (53–55), lung adenomas (56), lymphosarcoma of the lungs (49) and adenoma-like growths in the pituitary (50). A similar delayed appearance and reduced incidence was found for mammary tumors in rats maintained on a basic diet with intermittent fasting (26).

Tannenbaum and Silverstone (57, 58) reported a marked reduction in the incidence of and a delay in the time of appearance of spontaneous hepatomas after mice were maintained on a diet low, 8%, in protein *ad libitum* or on an isocaloric basis. The formation of these tumors appeared to be dependent upon the proportion of protein in the diet, but the diets permitted relatively good growth when compared to other diets containing higher proportions of protein. Although no such effect was found on the incidence of mammary carcinomas, there were indications that there was a delay in the time of their appearance.

In other experiments where mice were chronically underfed or restricted calorically on a supplemented commercial diet, Tannenbaum and Silverstone (59) found that an increased life span was accompanied by a decreased growth rate of mammary tumors and also by a lessened frequency of metastasis to the lungs.

High fat diets produced an increase in incidence and promoted an early appearance of spontaneous mammary tumors, but there was no effect upon primary lung tumors (60). A rather precise association between caloric intake, the incidence of and the time of appearance of spontaneous mammary and lung tumors in different strains of mice was also reported by Tannenbaum (61). This association became apparent when the caloric intake was restricted beyond 60% of *ad libitum* values, or by an over-all reduction in total intake of diet (56).

The dearth of comprehensive biochemical studies of the effects of the nutritional state or even of 'normal' aging is replicated in a search for information as to the lifelong relationships among enzymatic activity, age and diet. It is beyond the scope of this paper to cite the biochemical data from short-term nutritional studies.

Evidence of an adaptive metabolic mechanism to changes in the nutritional state is found in the observation that dietary protein requirements necessary to maintain nitrogen balance and total body nitrogen in the adult organism are less than those needed by young growing rats (62, 63). This is supported by the observation of Sharp *et*

al (64) that the rate of protein turnover in the young is one and one-half times that in older subjects. Furthermore the energy requirements for maintenance during chronic undernutrition is said to be decreased as well (32). Rosenthal and Allison (65) found that restriction in caloric content of diets fed adult rats, depleted body fat and protein, reduced nitrogen balance and increased catabolic activity, with a subsequent 'adaptation in metabolism' to maintain equilibrium.

The dependence upon the levels of other dietary factors for utilization of each essential component in a nutritional scheme, is recognized (66–69). Swift *et al.* (70) showed that the utilization of 'metabolizable energy' decreases with an increase above 25% of dietary protein, and the protein-sparing action of carbohydrates and of fats has been detailed in the report on the interrelationships of these three components by Yoshida *et al.* (71), by Munro & Wikramanayake (72), and by Bosshardt *et al.* (73). French (17), however, reports an inverse relation between life span and 'increased efficiency of utilization'. The limits within which this relationship holds are still to be determined. Sinclair (74, 75) brings the problem of delayed effects into being when he questions the desirability of overfeeding early in life.

Nutritional cirrhosis and production of fatty liver resulting from protein undernutrition, or diets low in essential amino acids, are reported by Farber (76) to be the result of an imbalance in the cell between protein and enzyme levels on the one hand and the cellular synthetic requirements on the other. The South African Bantu consumes a diet adequate in calories and in gross protein but low in animal protein and in fat, and maintains a low serum cholesterol level (of adults) and has little severe atherosclerosis of the aorta and coronary vessels (77). The death rate due to coronary heart disease is only $\frac{1}{10}$ that found in this country.

Horst *et al.* (78) found that young rats restricted in growth had a more youthful level of basal heat production than control rats of the same age.

Nelson (79), studying certain biochemical changes in rats receiving diets restricted in calories, found that moisture content of the fat-free carcass was no different than in the control rat, but relative protein content was similar to younger control rats of the same body weight. The ash content was also nearer that level found in chronologically younger rats. Increase in urinary albumin with increasing age in the 'control' rats was dependent upon the diet or its supplementation, and was nearly absent from the restricted animals.

The enzymatic approach to the study of aging is of relatively recent origin and studies have been confined mainly to the aged organism or to changes found in a few aspects of metabolism (80, 81). Shock and his associates (82) have reported variations in cholinesterase, oxygen uptake and other metabolic factors (83, 84) during aging but the effect of diet on these systems was not investigated. Knox *et al.* (85) have reviewed the literature dealing with the effects of environmental conditions, including diet, upon enzyme activity and have concluded that environment does affect enzyme activity and that there are adaptive mechanisms which bring the organism into a state of equilibrium with its new environment. Unfortunately the information cited relative to the dietary influences does not consider the age factor, and is in general obtained from short-term studies.

That environmental factors, whether internal or external in nature, must exert some effect upon enzymatic systems, directly or indirectly, seems undeniable. Examples can be seen in studies relating to frank nutritional deficiencies (86–88), or in the analysis of the action of substances of possible therapeutic value (89).

Long-term studies initiated in this laboratory 8 years ago indicated that an enzymatic approach to the study of nutritional effects upon disease processes and the aging phenomenon, might prove most fruitful. The sensitivity of enzyme activity changes in response to changes in environmental conditions, during the entire span of life, may possibly afford one means of attacking certain problems of interest to this symposium.

In our initial studies (90), 23 hepatic enzymes representing energy, oxidation and phosphate, carbohydrate and protein metabolism, were assayed during the first year of life of rats consuming a 'stock' diet. The majority, 18, of these enzymes increased in activity with age, but 4 decreased and 1 remained the same. It was reasoned that if enzyme activities were involved in the processes of growth, development and maturity, there should be compensatory changes in these metabolic systems in animals maintained on diets which, according to the work of others, resulted in increases or decreases of life span.

In the exploration of these thoughts, our

studies revealed that protein depletion of different aged rats altered the levels of those enzymes studied earlier, to that level found in chronologically younger rats (91). New activity levels induced by protein depletion were independent of the characteristic increasing or decreasing levels which normally accompany aging of these rats. It was thus found possible to produce a pattern of enzyme activity which was consistently like that found in young animals.

Four enzymes, alkaline phosphatase and cathepsin which decrease in activity with age, and histidase and d-amino acid oxidase which increase with age, were chosen for further study (92) in order to determine the effects of protein at different levels of feeding. It was found that for a given age, alkaline phosphatase activity decreased with increasing amounts of casein in the diet up to 30%, whereas histidase increased in activity under the same conditions (93). Additional casein in the diet produced an increase in activity of alkaline phosphatase and a decrease in activity of histidase. In these experiments the constituents of these diets, other than casein and sugar, were kept constant and were ample. An increase in casein content was made at the expense of the sugar content and the lowest activity values for a rat of any age were found when the casein and sugar were present in approximately equal proportions (93). Enzyme activities also were found to be directly affected by variation in the casein content when the sugar content was held constant, and by variation in the sugar content when the casein content was held constant. This study involved over 40 diets, in which series of isocaloric diets were compared and in which only the levels of protein and carbohydrate were varied. It was found that changes in caloric content only, affected the degree of change of enzyme activity and not the direction. The highest activity and the lowest activity resulting from the consumption of calorically similar diets was found to be directly and inversely related to the caloric content of the diet (93). The relative amount of protein or of carbohydrate in the diet appeared to be the determining factor for the level of enzyme activity for a rat of a given age, rather than the amount of food consumed.

In this diet-age-enzyme activity relationship, it was realized early that the enzyme activity of rats could be adjusted easily and precisely from the levels of one age to those of another age by the simple method of adjusting the diet. In these experiments the resultant enzyme changes from one age level to another were not accompanied by analogous weight changes.

It was also found that the relationship among enzyme activity, chronological age and dietary constituents was so well-defined that it was possible to predict enzyme activity levels provided the dietary make-up and the age of the rat were known. This suggested the existence of a 'pattern' of enzyme activity characteristic for any age and it was found that enzyme activity levels could be 'titrated' to any preassigned value.

Realimentation of rats on an experimental diet resulted in a return within 3 days to enzyme activity values characteristic of the rats' chronological age (94; Ross, unpublished observations). Studies of the rates of change in enzyme activity values (Ross, unpublished observations) after change in diet indicated that the major alterations in activity occurred within 24 hours after institution of a new diet. The rate of change then asymptotically approached its new level with time.

Nonenzymatic studies were carried out at the same time on the moisture and nitrogen contents, and changes in number, type and relative size of cells and nuclei (90, 91, 95) and on the mitochondrial content of hepatic cells (94, 96). As with enzymatic changes, these parameters also changed to values more like those found in chronologically younger animals when the proportion of protein to carbohydrate was reduced in the diet.

If alteration of enzyme activity patterns by manipulation of external environmental conditions, such as diet, does indeed influence disease incidence and life span, then it follows that biological and biochemical aging are synonymous, and any prolonged alteration in the biochemical make-up of an organism might affect all life processes. To test these hypotheses a long-term program involving more than 1000 male, Sprague Dawley rats was initiated in February of 1955.

The rats obtained from the Charles River Breeding Laboratories were derived from breeders vaccinated for *Salmonella enteritidis* and *S. typhimurium* and were specifically chosen for rapid growth rate and for relative freedom from disease. On arrival they were 35 to 40 gm in weight and were 21 days of age (3 days post-weaning). They were divided into 5 groups, housed individually and remained under observation and study until death.

One of the 5 groups of rats received the control diet (Laboratory Chow, Purina Co.) which was

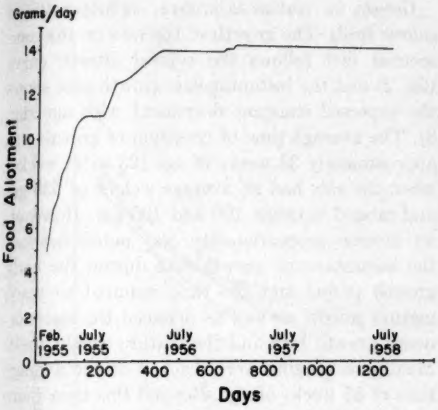


FIG. 1. Daily food allotments to rats maintained on the low casein-high sugar diet and to those on the high casein-high sugar diet. Estimation of daily allotments made on the basis of the consumption of rats given the low casein-high sugar diet, *ad libitum*. Grams food allotted daily to rats for a high casein-low sugar intake and a low casein-low sugar intake were 59% and 37%, respectively, of that amount of ration shown above.

fed *ad libitum*. The 4 experimental diets employed were designed so that the only dietary variable was the allotment of specific amounts of protein (Vitamin-free Casein, General Biochemical Corporation) and of carbohydrate (cane sugar). All rats in each of the 4 experimental groups were given each day a known amount of food, dependent upon their chronological age, in such a manner that the intake of all the constituents was identical within each group. The intakes of the experimental diets could be characterized as follows—*diet A*—high in protein and high in carbohydrate intake; *diet B*—high in protein and low in carbohydrate intake; *diet C*—low in protein and high in carbohydrate intake; *diet D*—low in protein and low in carbohydrate intake. In 2 groups similar protein allotments were made but the carbohydrate content was different. In the other 2 groups the allotments of protein were also identical to each other, though smaller in amount than in the former 2 groups, but the carbohydrate allotment to these 2 groups differed. The caloric intake of 2 of the 4 groups (*diets A* and *C*) was identical. In addition to the casein and sugar, every rat in all 4 groups received known and identical allotments of fat (Mazola Oil, Corn Products, Inc.), adequate amounts of vitamins (crystalline or in purified form supplied by Merck, Hoffman LaRoche and Lederle), minerals (U.S.P. XII) and trace elements.

The daily food intake of a 'pilot' group of rats which had been started earlier on diets low in casein and high in sugar content, determined the amount of food to be allotted to each of the 4 experimental groups of rats over their entire life span (fig. 1). This pilot group provided a basis of feeding because it had been found that the intake by rats on this diet was less than the intake by rats on the other diets under test, thus assuring complete consumption of allotted food. The precise amount of casein and of sugar given to each rat in each group was determined not only by the 'paired-group feeding' nature of this experiment (table 1), but by the increasing demands of chronological age and of size. The following table is a representative daily work sheet indicating the intake of the major components in each of the 4 experimental diets at the age period when rats on low casein-high sugar intake were allotted 10 gm of food per day:

TABLE 1. SAMPLE WORK SHEET: DAILY INTAKE PER RAT

	Diet A	Diet B	*Diet C*	*Diet D*
	HP[1]-HC[2]	HP-LC[3]	LP[4]-HC	LP-LC
	gm	*gm*	*gm*	*gm*
Casein	3.0	3.0	0.8	0.8
Sugar	6.1	2.0	8.3	2.0
Fat	0.5	0.5	0.5	0.5
Salts	0.4	0.4	0.4	0.4

HP[1] = High protein. HC[2] = High carbohydrate. LC[3] = Low carbohydrate. LP[4] = Low protein.

Two groups of rats received similar allotments of the isocaloric diets, *A* and *C*. The intakes of protein, of total food, and of total calories by the rats on the 4 experimental diets is shown in table 2. The 2 separate allotments in this table represent amounts given at 21 days of age (beginning of the feeding program) and from approximately 700 days of age until death.

Weekly examinations for external and possible internal abnormalities were made at time of weighing. Quarantine conditions were imposed on any rat when signs of disease became evident. At 7 different age periods between 3 weeks to 33 months, rats from each of the 5 groups were chosen at random from the total colony or from among the surviving population. These rats were sacrificed and a careful examination of external and internal structures was made. More

than 12 tissues were routinely removed from all rats. Tissues which appeared to have frank pathological lesions or suggested the possibility of being abnormal were also secured. These tissues are presently available for histopathological studies to be performed in order to determine the age-specificity of internal disease during the entire life span of the rat and the extent to which these age specific incidences are affected by diet.

Cytological studies of livers from rats sacrificed for the biochemical assays are to be made to determine the effect of specific diets on the changes in number and proportion of cell types, relative cellular and nuclear sizes and their nucleocytoplasmic ratios, during aging. The quantitative biochemical assays, made according to standard procedures and reported earlier (89–92), include the activity of liver homogenates of catalase, adenosinetriphosphatase, histidase and alkaline phosphatase and hepatic nitrogen, lipid and moisture content. Growth, developmental characteristics, body proportions, gross pathological and mortality records were also kept. The quantitative nonenzymatic values will also be used as additional bases for computing enzyme activity.

Some of the highlights of this as yet unfinished work are presented here, but the full results and conclusions must await completion of the work.

TABLE 2. DIETARY ALLOTMENTS

Diet	Initial			Maximal		
	Total food	Total calories	Casein	Total food	Total calories	Casein
	gm		*gm*	*gm*		*gm*
A	4.9	20.0	1.47	14.3	58.5	4.29
B	2.9	12.0	1.47	8.5	34.8	4.29
C	4.9	20.0	0.39	14.3	58.5	1.14
D	1.8	7.6	0.39	5.3	22.5	1.14

Growth in relation to protein, carbohydrate and caloric levels. The growth of the rats on the commercial diet follows the typical growth curve (fig. 2) and the instantaneous growth rate shows the expected constant decrement with age (fig. 3). The average time of cessation of growth was approximately 33 weeks of age (23 to 44 weeks) when the rats had an average weight of 530 gm and ranged between 400 and 700 gm. However, an inverse proportionality was noted between the instantaneous growth rate during the early growth period and the time required to reach mature weight as well as between the instantaneous growth rate and the mature weight itself. Maximum weights were reached at the average time of 85 weeks of age. Beyond this time there was a gradual weight loss until death, and this loss in weight as well as the death-weight was found to be directly proportional to the adult weight or to the maximum weight of these control rats.

The growth of the rats on experimental diets was considerably restricted as compared to the controls (fig. 2). A comparison of the early *ad libitum* intake of other rats on the same 4 diets in the same age periods, with the intake of the rats in the experimental groups, showed approximate restrictions of intake of the order of 42%, 68%, 0% and 80% for *diets A, B, C* and *D*, respectively. Similarly, a comparison of the average weight of the rats of these groups with the average weight of those on *ad libitum* intake shows a reduction of 14%, 63%, 0% and 78% for *diets A, B, C* and *D*, respectively. The decrease in mass, therefore, appears to be a function of food restriction.

Weanlings on *diet D* which was markedly restricted in protein and calories, not only maintain themselves but increase in size, once they pass through an 'adaptation' period. Even though the amount of food allotted each rat in each group was held constant for a considerable period

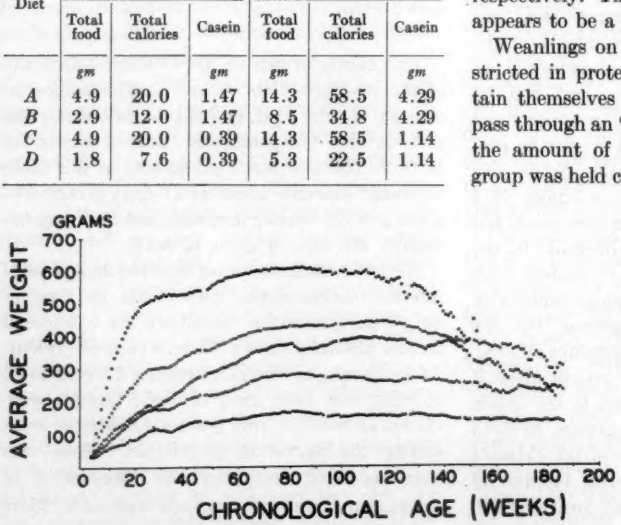


FIG. 2. Influence of diet on growth of rats and subsequent weight changes with age. ● = Purina lab Chow; ○ = high casein-high sugar intake; △ = high casein-low sugar intake; □ = low casein-high sugar intake; ▲ = low casein-low sugar intake.

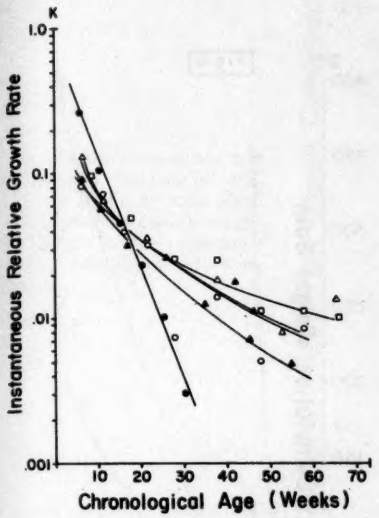


FIG. 3. Comparison of changes in instantaneous relative growth rates of rats on different diets. $K = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$; W = weight in grams, t = time in weeks. See fig. 2 for key to symbols.

of time after the 60th week, mass-accumulation continued in all experimental groups. Even at 700 days of age when the maximum daily allotment was reached (table 2), the trend toward increase in mass is still evident except for rats maintained on *diet B*. The maximum weight reached by the rats on all experimental diets was considerably less than that of the controls and the age at which it was reached differed for each diet. The changes in instantaneous growth rate for these rats are different from those found for the control group (fig. 3). On experimental diets the decrease in rate with age is slower and of a more complex nature and in general is inversely proportional to the chronologically increasing intake of food (fig. 1) for the first 20 weeks. These animals as well as the controls showed a slow but steady decline in weight after reaching a maximum, and death-weight was directly proportional to the maximum weight in all groups. This proportionality between maximum weight and death-weight is not only a direct one, but forms a continuum when a plot is made of values from rats on all 5 diets.

After 6 months of life, the positive effect of calories on the weight of rats on experimental diets becomes apparent and is influenced by the level of casein intake. Because the rats on these experimental diets never reach sizes comparable to those on the control diet, an assessment of dietary effects can be made only on the overall growth picture for each group or for any one given period. This increase in mass ceases for each group at different time periods and, as noted earlier, signals subsequent loss in weight. It would appear from the relationship between cumulative total caloric consumption and average maximum weight that the weight was not only influenced by caloric intake but is predictable (fig. 4 and 5). The caloric intake of rats on the high casein-high sugar diet and of the rats on low casein-high sugar, are identical but increase in weight ceases earlier on the latter diet. When maximum weight gain is plotted against protein consumption, the relationship seen in figure 4 is not found. However, as seen in figure 6, more than 2 times the amount of protein is required by animals on the high casein-high sugar diet to reach the same weight reached by the rats on the low casein-high sugar diet, prior to their maximum weight.

The efficiency of utilization of casein is shown in figures 7 and 8. After 1 year of life, rats on *diets A*, *B* and *D* have quite similar efficiencies of utilization of casein. Rats on *diet C*, however, have remarkably greater efficiency of utilization up until 2 years of age, at which time this diet in the amount given is inadequate for further growth.

An indication of the influence of the state of nutrition on growth processes and on the efficiency of conversion of food substances into mass is seen from the analysis of the amount of casein and of sugar required to increase the weight of growing rats from 50 to 100 gm. Identical

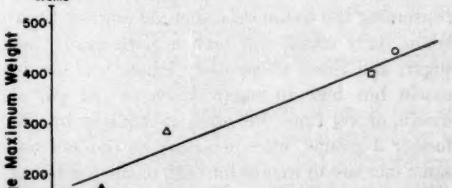
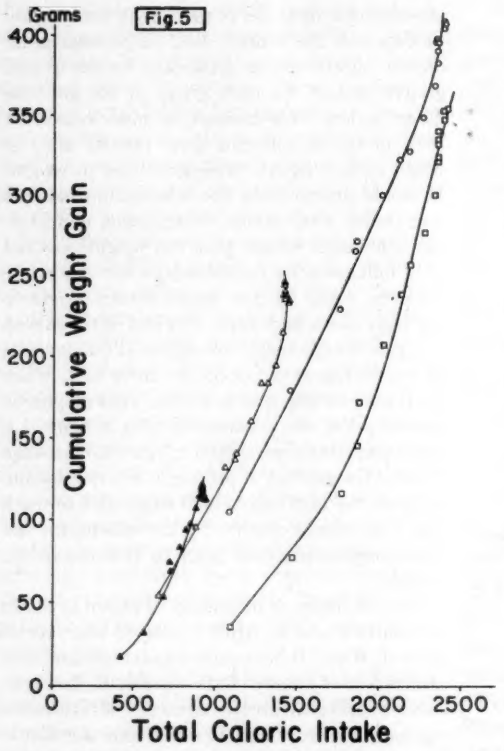


FIG. 4. Linear relationship between maximum 'growth' weight of rats and cumulative caloric intake. The time necessary to reach average maximum weight differed for each group. See fig. 2 for key to symbols.



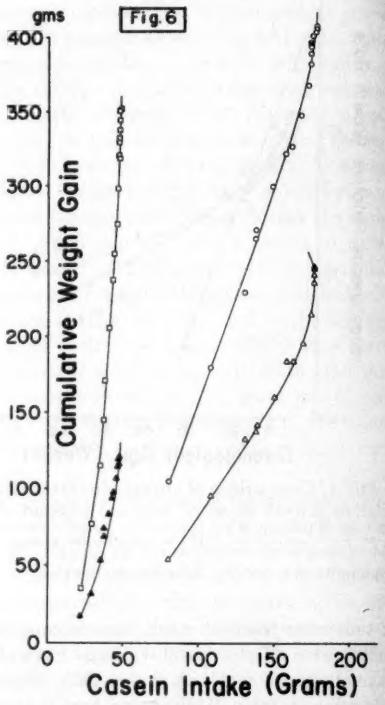


FIG. 5. Dependence of cumulative weight gain on caloric content and intake by rats of different experimental diets. Each point represents gain in weight and total caloric intake within successive 6 week periods. See fig. 2 for symbols.

FIG. 6. Dependence of cumulative weight gain on casein content and intake by rats of different experimental diets. Each point represents gain in weight and total casein intake within successive 6 week periods. Time periods to reach maximum weight were 114, 84, 108 and 78 weeks and the cumulative casein intake to reach maximum weight during these periods were 3014, 2123, 757 and 518 grams for ○, △, □, and ▲, respectively. See fig. 2 for symbols.

amounts of casein, 33 gm, were required to double the weight in 3 of the 5 groups of rats: those consuming the commercial diet, *ad libitum*, those whose daily intake was high in both casein and sugar, and those whose daily intake was low in casein but high in sugar. However, 82 gm of casein, or 2½ times the amount required by the former 3 groups, were necessary to produce the same increase in weight for both of the remaining groups of rats—those whose daily intake was high in casein and low in sugar, and those whose daily intake was low in both casein and sugar. Not only is more casein necessary for this gain in weight but the cumulative caloric consumption is higher for those rats whose daily intake of casein is low without regard to the daily intake of sugar. Although lean body weight is not considered in these calculations, these data may be of interest to those concerned with the economics of animal production.

Hepatic enzymatic activity in relation to the nutritional state and aging. For approximately the first year of life of rats maintained on commercial diet, it was found that catalase, adenosinetriphosphatase and histidase increased in activity while alkaline phosphatase decreased in activity. Beyond 300 days of age, a reversal in direction of activity of all 4 enzymes took place and the age at which this reversal in direction occurred was approximately the same for all rats in this group, and the relative change was the same for each enzyme (fig. 9). This reversal occurred approximately at the time these rats reached a mature weight but considerably before

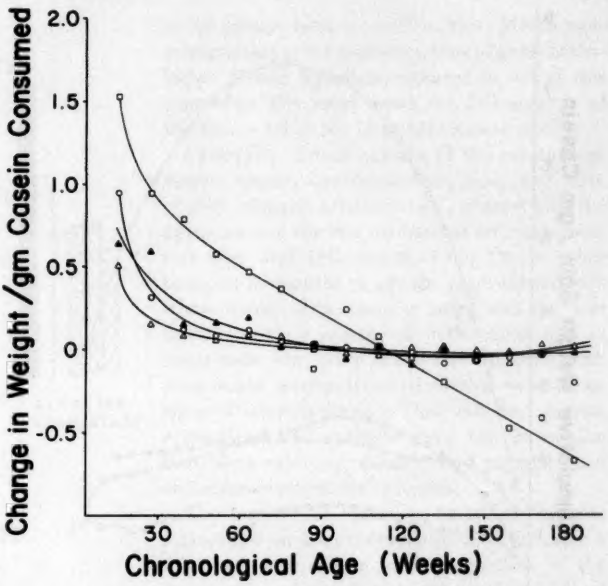


FIG. 7. Influence of age and of diet upon efficiency of utilization of casein by rats. Each value represents gain in weight per gram casein consumed within successive 12-week periods.

maximum weight had been attained. During senescence minor discrepancies in certain enzyme activity values of individual rats were found and these may have been due to the effects of internal disease superimposed upon the aging processes.

Rats maintained on a diet which alters the enzyme activity levels to that found at an earlier age, should have a correspondingly increased life span. It was found that enzyme activities in those groups of rats maintained on the 4 semi-synthetic diets differed not only in the rate and degree of change during aging but also as to the time of their reversal of direction of activity, or transition period, and as will be shown later correlated with life span.

The general scheme of the changing direction of enzyme activities (i.e. increase followed a decrease of activity for catalase, adenosinetriphosphatase, histidase and a decrease followed by an increase of activity for alkaline phosphatase) followed that found in rats on the commercial diet. In general, the rate and degree of change of enzyme activities in the experimental groups appeared to correlate with the levels of both the casein and sugar intake. The greatest change in activity from one time period to another was found in those animals maintained on a diet ample in both the dietary constituents under study. As 2 enzymes and 2 nutritional states are representative of results found for the other enzymes and nutritional regimens in this experiment, the data obtained on alkaline phosphatase and adenosinetriphosphatase in those rats maintained on the control diet and on the low casein-high sugar diet (*C*) are shown in figure 10.

The rate of change in alkaline phosphatase activity for the rats maintained on the low-casein diet lags behind that found in the control group. The lowest activity prior to the point of inflection on the curve occurs at 700–800 days of age for the experimental group as contrasted with 250–300 days in the control group. In a similar manner, adenosinetriphosphatase, whose direction of change in activity with age is opposite to that of alkaline phosphatase, increases for approximately 700–800 days, and then it too reverses in trend. The same delay in reversal or point of inflection was noted in general for the values of other enzymes.

The activities found in the group of rats allotted *diet A* which was identical calorically to the above-mentioned diet (*C*), but which differed only as to the intake of casein, showed a similar pattern of enzyme change. The degree and rate of change and eventual time of transition of their enzyme patterns were, however, different. The same general picture was found, with the exception as to degree and rate of change and transition time for those rats on *diets B* and *D*.

The changing enzyme activity values with age

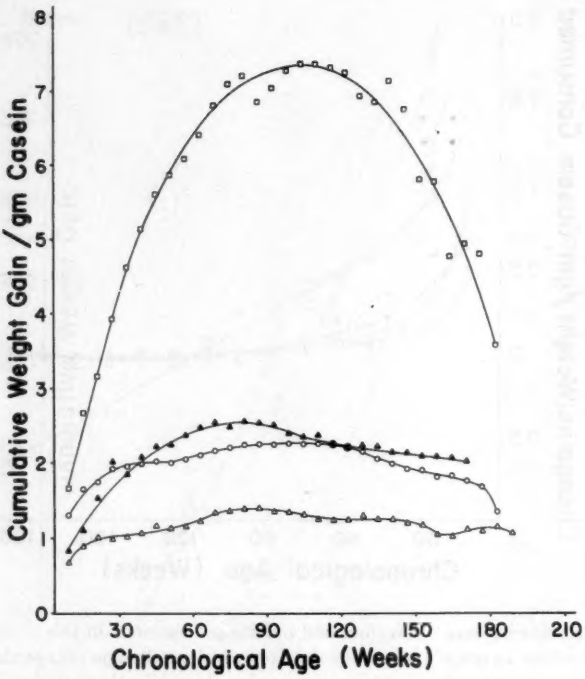


FIG. 8. Comparison of effect of diet and of age of rats upon cumulative weight gain per gram casein consumed. Each point represents successive 6-week periods.

and the reversal in direction of these changes were also found in the values for hepatic nitrogen, lipid, moisture content and in organ weight-body weight ratios. Furthermore, the time of transition of these values for each of 5 groups of rats is the same as found for the enzymes in each respective group. The per cent total nitrogen and lipid content increases in value at first and then decreases with increasing age, while per cent moisture content and ratio of organ to body weight decreases and then increases.

It is apparent that the levels of hepatic enzyme activity and of other biochemical processes are influenced by the nutritional state and that this influence is not limited to one system. When a change is found in one enzyme system within a group, compensatory changes are noted in an opposite direction in the others, regardless of the fact the average value of one may be increasing and those of others decreasing with increasing age.

With increasing age, biochemical patterns evolve which are characteristic of each dietary group, and the effect of the different dietary states may be one of tempering or enhancing the rate of development or flux of these patterns. Not only does this pattern exist for the different experimental groups but the same conclusions may be drawn for each sacrificed individual within the group. A comparison of the levels of enzyme activity from one individual to another of the same age, nutritional background and environment, shows expected variations in value within each parameter, and the extent of variation for any one assay increases with age. But there appears to be an uncommon uniformity of pattern of total biochemical parameters within each individual. When there is a 'deficiency' in one enzyme level when compared to the values of the group (for a given time period or diet) there are compensatory increases in activity of other systems within that individual. Such an integration of the available biochemical data shows a remarkable cohesion and stability of pattern values from one animal to another, one group to another and one age to another. It would appear that there is a greater biochemical similarity between organisms on this basis, than one would be led to expect on the basis of comparison of one part or assay of one organism to another. This 'compensatory balance' is evident regardless of diet except in those animals suffering from frank degenerative disease processes. The presence of an over-all biochemical imbalance

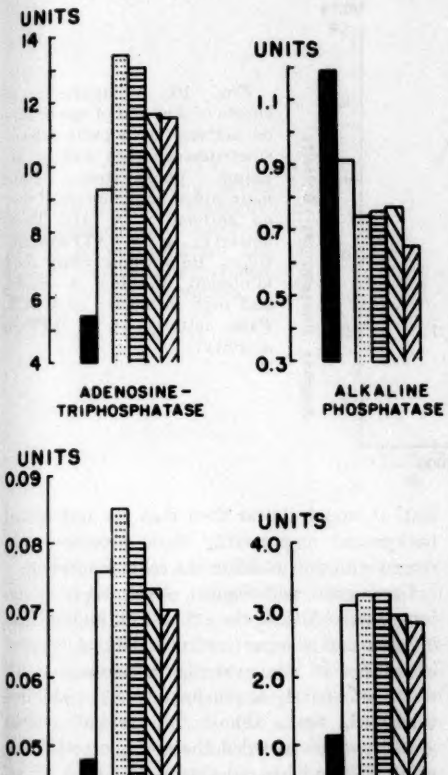


FIG. 9. Hepatic enzyme activity changes at different ages. Rats maintained on commercial ration, *ad libitum*. Age at sacrifice: ■ = 21 days; □ = 120 days; ▨ = 220 days; ■ = 385 days; ▩ = 600 days; ▨ = 795 days.

which may be indicative of a loss of adaptability, during a disease state, could well contribute to the termination of the organism's life.

Influence of protein and calories on organic disease. If the metabolic pattern of an organism, which is affected by its state of nutrition, is an influencing factor in the etiology of organic disease, then the rate of development, the time of appearance of recognizable physiological and structural changes, and the incidence and severity of age-associated diseases should be different in animals whose nutritional regimens during their entire lives were different. Observations on the state of health and of the grossly recognizable presence of degenerative diseases in the animals in all groups tend to confirm this. Microscopic confirmation of these observations of gross pathological lesions cannot be reported in full at this time since this must await the full analysis of the tissues taken for histopathological studies.

Cataracts, chronic eczema of the extremities, coarse, sparse, discolored hair, scaly dry skin, obesity, sluggish attitudes, etc., characterize the appearance of the rats maintained on commercial diet after their 18th month of life. On the other hand, at 30 months of age the experimental rats whose intake of casein or of sugar was low, and especially when intakes of both casein and of sugar were low, were clean, had fine hair with thick coats, were active and showed no external signs of internal disease. Their size and general appearance and attitude gave the impression that these rats were considerably younger than their chronological age indicated.

The number of cataracts found in the rats maintained on the experimental diets indicates a relationship to caloric intake regardless of the composition of the diet. Approximately 15% of the animals whose total caloric intake was high (*diets A* and *C*) had corneal opacities but only a few had them whose caloric intake was lower, and there was a negligible incidence in the severely restricted rats.

Dermatological conditions were found in greater number in the control group and in those rats whose intake of casein was high without regard to the sugar intake. Only a negligible number of cases was found in those whose intake of casein was low.

The rats whose intake of both casein and of sugar was low were prone to licking their thick hair coats, especially during the warmer seasons of the year even though ambient animal room temperature was maintained between 75°–78° F the year round.

Forty per cent of the rats still alive after one year, which were maintained on the commercial diet or those whose intake of both casein and sugar was high (*diet A*), developed spontaneous tumors. Sites of development included skin, eyes, mammary areas, pancreas, liver, intestine, spleen, kidney, testes and prostate. Among those tissue sections which have been examined microscopically, fibromas, sarcomas, adenomas and carcinomas have been found.

In contrast, only 20% of the rats whose intake of casein was low, but which had a high sugar intake (*diet C*), developed tumors. It should be emphasized that the caloric intake of these rats

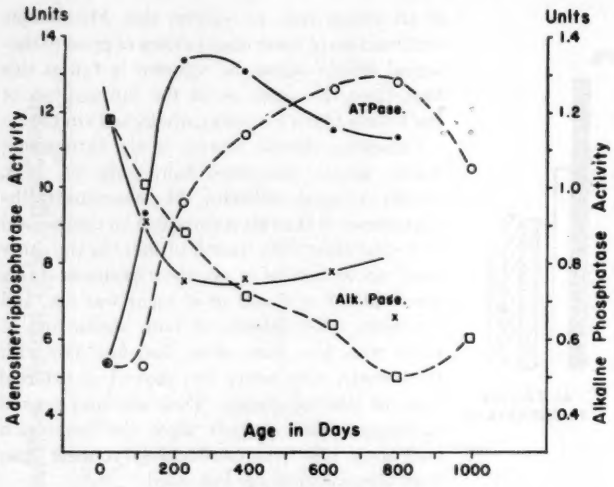


FIG. 10. Comparison of effects of diet and of age of rats on activity of hepatic adenosinetriphosphatase and of alkaline phosphatase. Rats maintained on commercial diet *ad libitum*: × = Alk. Pase. activity; ● = ATPase activity. Rats whose daily food allotment was low in casein and high in sugar: □ = Alk. Pase. activity; ○ = ATPase activity.

was identical to that of those on the high casein-high sugar diet (*A*), but the latter group had twice the number of spontaneous tumors. Rats on *diet B* (high casein-low sugar) developed tumors to the extent of only 15% while rats on *diet D* have so far shown an incidence of only 2%.

The time of appearance and the age-associated distribution of these tumors in the various groups also differed. A positive correlation does exist, however, between the incidence, the time of appearance and the enzymatic transition period. The longer the enzymatic transition period was delayed, the longer the rats were free of tumors and there were fewer tumors developing subsequently. The influence of diet is further noted in the direct relationship found to exist between the average growth rate of a population and the incidence of tumors. Initial analysis of individual growth rates within each population indicated that rats with relatively high growth rates early in life may have a greater chance later in life of developing a tumor than do those rats whose early growth rate was relatively lower.

Another indication of the effects of specific long-term nutritional states upon neoplastic processes is illustrated in an attempt to determine whether the nutritional level of protein and carbohydrate was too low in the rats maintained on *diet D* to support a tumor if one did develop. It was found, in another group of rats maintained on *diet D*, that a transplantable tumor, Walker 256, could be successfully transplanted to rats under this nutritional regimen as well as to those rats maintained on diet A or on the commercial diet. It would appear then that the nutritional background may modify those processes concerned with the initiation of a cellular accident.

Upon gross pathological observations at autopsy, some 40% of the animals on intakes high in casein and in sugar (control and *diet A*) showed indications of cardiovascular abnormalities. Of those on *diet A*, approximately 8% exhibited thickened, rigid, almost 'pipe stem' arterial systems which included the aorta, carotid, brachial, iliac and femoral arteries. No such abnormalities were found in the other experimental groups even though the type and the amount of fat consumed by each rat each day was identical.

Other cardiovascular lesions such as hypertrophy of the heart and varicosities particularly of the mesenteric vessels, were found in considerably smaller number. Rats whose intake was isocaloric with *diet A* showed only ⅛ the incidence of those on *diet A*, and none was found in rats whose intake was low in both casein and sugar. A similar relationship was found between the incidence of renal abnormalities and the state of nutrition. Renal lesions were found in 75% of the rats on the control diet and on *diet A*, after one year of age, and this incidence was not only higher than those found in the other experimental groups but the lesions were considerably more severe.

The incidence of respiratory disease over the entire life span was uniform in each population and not appreciably affected by diet, but the disease process was more severe in the rats on commercial diet or on *diet A*.

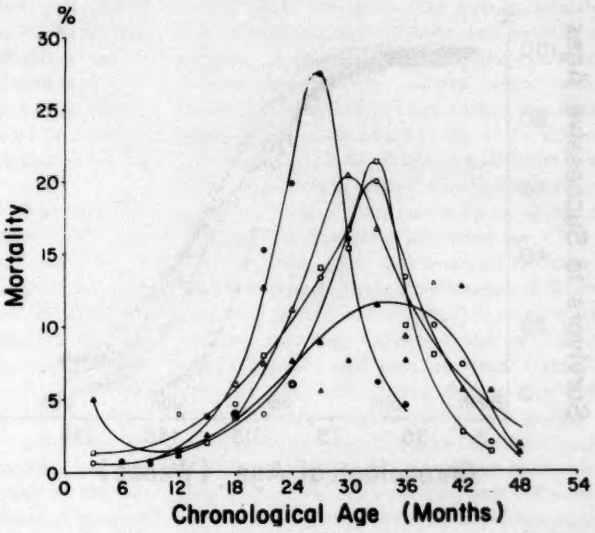


FIG. 11. Influence of diet on mortality distribution-age pattern. Rats dying of accidents and those sacrificed for biochemical studies not included. See fig. 2 for key to symbols.

Gastrointestinal affections such as ulcers and thickenings of the mucosa were found in all groups to a limited degree. Rats on *diet D* were especially prone to caecal impactions during the first 6 months of their lives, and during the 3rd year of life these rats had a very high incidence of trichobezoar formation. The cause of death in this group past the first 20 months of life, in most instances, could be ascribed to the chronic effects of the presence of hairballs in the stomach.

Abnormalities of male sex organs were found to be principally hypertrophy, tumor formation and atrophy. These conditions were found late in life in rats having high casein consumption. In those rats whose intake of casein was low, no obvious pathology of male sex organs was found if the rats did not suffer from debilitating diseases. Microscopic examination of the testes obtained from the rats sacrificed for biochemical assays showed a delay in active spermatogenesis in those rats whose intake was low in both casein and sugar, but at 1000 days of age the rats in this group still showed sperm formation.

Influence of protein and calories on life span. The assessment and correlation of these data becomes a difficult and complex task inasmuch as the consideration of type, incidence and severity of the organic disease process, the over-all physical and functional activity and the possible changing nutritional needs during life, must be taken into account. Although the information necessary to make conclusive statements is not yet at hand, certain correlations may now be attempted. Only the corrected mean and the mode of life span and the mortality distribution of the various populations will be considered since the studies and their analysis have not been completed.

The average life span of the control rats is considerably longer than life spans cited by early workers and is in agreement with more recent reports. The average life span of the control rats permitted to live out their lives was 729 days, the last rat in this population dying at 1072 days of age. The mortality distribution shown in figure 11, reveals a mode of 26 months (780 days) of age. The curves of mortality distribution and of per cent survival time to successive ages for each of the experimental groups are significantly displaced toward older ages as compared to the control group (see figs. 11 and 12). The average life span of 840 days or the mode of more than 900 days for the group of rats maintained on a low casein-high sugar intake (*diet C*) is the result of a reduction in mortality rate after the first year of life. The longest-lived rat in this group died at 1300 days. Comparison of these 2 groups in figures 10 and 11 shows the close correlation between enzymatic pattern changes and prolonged life span. The greater increase in life span of rats on the other diets also correlate, with some minor exceptions, with the level of enzyme activity, its rate of change, and especially with the chronological age when the transi-

FIG. 12. Comparison of survival rates of rats maintained on different diets. Final values for the rats on diets △ and ▲ not available at present as some rats still surviving. See fig. 2 for key to symbols.

tion of the enzymatic and nonenzymatic biochemical parameters occurs. From the present evidence, the longest-lived group will probably be those rats which had been and still are being maintained on the relatively high casein-low sugar intake (*diet B*) and will average more than 925 days. Some of the individuals in this group are now past 1400 days of age. Those rats maintained on intakes high in both casein and in sugar (*diet A*) lived an average of 903 days, the longest living 1300 days. The average life span of those rats with low casein and sugar intake (*diet D*) will probably be 850 days because of the number of deaths early in life from caecal impactions.

The food offered the experimental rats apparently was ample in quantity as well as in quality inasmuch as rats in these groups outlived those on the commercial diet which was designed for maximal growth. The data show that growth retardation over the entire life span does not correlate with average life expectancy, but such a correlation does exist for maximum size if the deaths during the first 2 years are ignored. With this correction, rats with both low casein and sugar intake (*diet D*) would outlive all other groups, having an average life span of better than 1000 days, rats on the high casein-low sugar diet (*B*) being next with an average life span of approximately 900 days. This might well mean that these experimental diets exerted their effects late in life. The significantly different 'uncorrected' average life spans of the 2 groups of rats allotted identical amounts of the isocaloric *diets A* and *C* mitigates against a correlation between caloric intake and life expectancy. On a corrected basis, however, such a correlation does exist since the life expectancies of rats in these 2 groups, after 2 years of age, are similar.

A consideration of the effect of the level of protein indicates that while relatively high protein intake during an entire lifetime is advantageous as regards average life span, a life time of low protein intake results in a reduction in mortality rate for the latter half of life.

COMMENTS

The lifetime feeding of different levels of protein and of carbohydrate in otherwise adequate diets has been shown, even prior to the completion of this experiment, to be an influencing determinant in the length of life. This is manifested not only in the mortality data but also in the incidence and time of development of organic disease. Two interpretations, using the available information obtained in these and in other studies relevant to the metabolic changes during aging, and as they are affected by diet, present themselves—one assumes an adaptation phenomenon, the other a heritable predisposition. The induction of a specific metabolic pattern by a protracted state of 'overnutrition' may be one major, underlying cause of organic disease and of a relatively short life span. Secondly, the metabolic patterns unique to each living system, which control and characterize growth, development and other physiological processes, may be pre-

disposing factors for the initiation and rate of development of age-associated pathological processes and for variation in the length of life. It would seem to be a logical conclusion that these basic control mechanisms are in a most fundamental manner retarded, enhanced or modified within limits by the nutritional state of the living system.

A relatively short life span and a high incidence of age-associated diseases are commensurate with overnutrition. However, severe undernutrition of protein or of calories or of both produces more desirable effects later in life, while its ability to do so early in life is questionable. The data obtained in these studies are highly suggestive that the over-all biochemical patterns, rates and transition periods, which mirror the organism's capacity to utilize available food substances for continuation of growth, are associated with life span. The need for a re-evaluation of the parameters used in determining levels and limits of nutritional requirements is indicated by the relationships that have been found in these diseased and nondiseased animals.

Of more importance than the conclusions that have been obtained from these studies are the questions one is now able to ask. Does the nutritional state early in life affect later needs, as well as later physiological and pathological processes? Is a maximum growth rate optimal for long life with relative freedom from organic ailments? How much protein, calories or any other nutritional factor is necessary, and in what relationship to each other, at different periods in life for a productive and prolonged life span? Are the specific nutritional needs of the individual affected not only by his immediate environment and state of development and genetic constitution, but also by his earlier nutritional background? Is the enzyme transition period a measure of the onset of senescence with its attendant disease processes, and is it a demonstration of a 'natural' shift of balance between anabolic and catabolic activities?

SUMMARY

Life expectancy of rats has been modified by nutritional means and the associated biochemical and pathological changes have been investigated.

Initial studies revealed that the levels of protein, of carbohydrate, and of calories in otherwise adequate diets, affected hepatic enzyme activities as well as the values of certain nonenzymatic assays. The enzymes studied represented those associated with energy, oxidation and phosphate, carbohydrate and protein metabolism. At any age these enzymes exhibited a characteristic activity pattern which was a function not only of the age, but of the dietary regimen. By means of alteration of the amounts and proportion of the dietary constituents it was found that levels of activity of individual enzymes could be changed from values found at one age to those characteristic of any other age.

A long-term study was initiated in order to determine quantitatively the influence of different intakes of protein and of carbohydrate on enzyme activity patterns, incidence and severity of organic disease. and upon life span. Over 1000 weanling rats were studied during their entire life span. They were divided into 5 groups according to the assigned intake of protein, carbohydrate and calories. Morphological, biochemical, pathological and actuarial data were collected.

The rates and degree of change of biochemical assay values with increase in age were found to be dependent upon the nutritional state. At different time periods, depending on the diet, these biochemical values, whether increasing or decreasing with age, showed a reversal in direction. The time when this transition occurred differed for each group and was found to be associated with specific developmental processes, subsequent increased incidence, type and severity of age-associated organic diseases, gross senescent changes and mortality rates. Observations relative to the effect of different life-long intakes of protein, of carbohydrates and of calories on spontaneous neoplasia, kidney and cardiovascular lesions are presented. Correlations among growth, development, senescent characteristics and lifetime nutritional history are discussed.

The lifetime feeding of different levels of protein, of calories, and of carbohydrate in otherwise adequate diets has been shown to be an influencing determinant or a modifying factor in the incidence of degenerative disease and in the length of life.

REFERENCES

1. Fenton, P. F. and J. M. Marsh. *J. Nutrition* 60: 465, 1956.
2. Palmer, L. S., C. Kennedy, C. E. Calverley, C. Lohn and P. H. Weswig. *Minn. Agr. Exper. Sta. Bull.* 176, 1946.
3. Driscoll, S. G. and D. Y. Hsia. *Pediatrics* 22: 785, 1958.
4. Leathem, J. H. In: *Hormones and the Aging Process*, edited by E. T. Engle and G. Pincus. New York: Acad. Press, 1956, pp. 79–97.

5. LEATHEM, J. H. *Recent Prog. in Hormone Research* 14: 141, 1958.
6. RUSSELL, J. A. AND A. E. WILHELMI. *Ann. Rev. Physiol.* 20: 43, 1958.
7. ARMSTRONG, D. B., I. DUBLIN, M. WHEATLEY AND H. MARKS. *J. A. M. A.* 147: 1007, 1951.
8. KATZ, L. N., J. STAMLER AND R. PICK. *Fed. Proc.* 15: 885, 1956.
9. WEISS, T. J. AND K. F. MATTIL. *J. Am. Oil Chemists' Soc.* 34: 503, 1957.
10. BROCK, J. F. *Ann. Rev. Biochem.* 24: 523, 1955.
11. GOODMAN, J. I. *Geriatrics* 13: 359, 1958.
12. WALKER, A. R. P. *Ann. New York Acad. Sc.* 69: 989, 1958.
13. LANE, P. W. AND M. M. DICKIE. *J. Nutrition* 64: 549, 1958.
14. SILBERBERG, M. AND R. SILBERBERG. *Am. J. Physiol.* 177: 23, 1954.
15. SILBERBERG, M. AND R. SILBERBERG. *Physiol. Rev.* 35: 347, 1955.
16. SILBERBERG, R. AND M. SILBERBERG. *Can. J. Biochem. & Physiol.* 33: 167, 1955.
17. FRENCH, C. E., R. H. INGRAM, J. A. URAM, G. P. BARRON AND R. W. SWIFT. *J. Nutrition* 51: 329, 1953.
18. KENNEDY, G. C. *Brit. M. Bull.* 13: 67, 1957.
19. GOLDBLATT, H. *Physiol. Rev.* 27: 120, 1947.
20. WAXLER, S. H., P. TABAR AND L. R. MELCHER. *Cancer Res.* 13: 276, 1953.
21. COHN, C., D. JOSEPH, L. OLINER AND J. V. MORTON. *Endocrinology* 62: 251, 1958.
22. JONES, R. J. AND S. HUFFMAN. *Proc. Soc. Exper. Biol. & Med.* 93: 519, 1956.
23. RUSSELL, W. C., M. W. TAYLOR AND J. M. HOGAN. *Arch Biochem. and Biophys.* 39: 951, 1941.
24. KORENCHEVSKY, V. *Texas Rep. Biol. & Med.* 12: 1006, 1954.
25. LANSING, A. I. In: *Cowdry's Problems of Ageing*, edited by A. I. LANSING. Baltimore: Williams & Wilkins, 1952, pp. 3–22.
26. CARLSON, A. J. AND F. HOELZEL. *J. Nutrition* 31: 365, 1946.
27. CARLSON, A. J. AND F. HOELZEL. *J. Nutrition* 34: 81, 1947.
28. MCCAY, C. M., M. F. CROWELL AND L. A. MAYNARD. *J. Nutrition* 10: 63, 1935.
29. MCCAY, C. M., W. E. DILLEY AND M. F. CROWELL. *J. Nutrition* 1: 233, 1929.
30. SLONAKER, J. R. *Am. J. Physiol.* 98: 266, 1931.
31. MCCAY, C. M., L. A. MAYNARD, G. SPERLING AND L. L. BARNES. *J. Nutrition* 18: 1, 1939.
32. MEYER, J. H., C. E. LUEKER AND J. D. SMITH. *J. Nutrition* 60: 121, 1956.
33. SCHULTZE, M. O. *J. Nutrition* 56: 25, 1955.
34. JACKSON, C. M. *The Effects of Inanition and Malnutrition upon Growth and Structure.* Philadelphia: P. Blakiston's Son and Co., 1925.
35. JACKSON, C. M. *Am. J. Anat.* 58: 179, 1936.
36. JACKSON, C. M. *Anat. Rec.* 68: 371, 1937.
37. QUIMBY, F. H. *J. Nutrition* 36: 177, 1948.
38. VAN DAM-BAKKER, A. W. I., A. P. DEGROOT AND R. LUYKEN. *Brit. J. Nutrition* 12: 259, 1958.
39. RIESEN, W. H., E. J. HERBST, C. WALLIKER AND C. A. ELVEHJEM. *Am. J. Physiol.* 148: 614, 1947.
40. TANNENBAUM, A. *Cancer Res.* 5: 609, 1945.
41. RUSCH, H. P., B. E. KLINE AND C. A. BAUMANN. *Cancer Res.* 5: 431, 1945.
42. LEE, Y. C. P., M. B. VISSCHER AND J. T. KING. *J. Gerontol.* 11: 364, 1956.
43. SHERMAN, H. C. AND H. L. CAMPBELL. *Proc. Natl. Acad. Sci.* 21: 235, 1935.
44. GYÖRGY, P. *Am. J. Clin. Nutrition* 2: 231, 1954.
45. ASCHKENASY, A. *Am. J. Clin. Nutrition* 5: 14, 1957.
46. SIMMS, H. S. AND B. N. BERG. *J. Gerontol.* 12: 244, 1957.
47. MCCAY, C. M. In: *Cowdry's Problems of Ageing*, edited by A. I. LANSING. Baltimore: Williams & Wilkins, 1952, pp. 139–202.
48. SAXTON, J. A., JR. AND G. C. KIMBALL. *Arch. path.* 32: 951, 1941.
49. BERG, B. N. AND C. R. HARMISON. *J. Gerontol.* 12: 370, 1957.
50. SAXTON, J. A., JR. *Biol. Symp.* 11: 177, 1945.
51. HENSCHEN, F. *J. Gerontol.* 8: 1, 1953.
52. MALMROS, H. *Acta med scandinav.* 246: 137, 1950.
53. TANNENBAUM, A. AND H. SILVERSTONE. In: *Advances in Cancer Research*, edited by J. P. GREENSTEIN and A. HADDOW, New York: Acad. Press. 1953, vol. *I*, pp. 451–501.
54. VISSCHER, M. B., Z. B. BALL, R. H. BARNES AND I. SIVERTSEN. *Surgery* 11: 48, 1942.
55. WHITE, F. R., J. WHITE, G. B. MIDER, M. G. KELLY AND W. E. HESTON. *J. Nat. Cancer Inst.* 5: 43, 1944.
56. TANNENBAUM, A. *Am. J. Cancer* 38: 335, 1940.
57. TANNENBAUM, A. AND H. SILVERSTONE. *Cancer Res.* 7: 711, 1947.
58. TANNENBAUM, A. AND H. SILVERSTONE. *Cancer Res.* 9: 162, 1949.
59. TANNENBAUM, A. AND H. SILVERSTONE. *Cancer Res.* 13: 532, 1953.
60. TANNENBAUM, A. *Cancer Res.* 2: 468, 1942.
61. TANNENBAUM, A. *Cancer Res.* 2: 460, 1942.
62. JACKSON C. M. *J. Nutrition* 13: 669, 1937.
63. HARTSOOK, E. W. AND H. H. MITCHELL. *J. Nutrition* 60: 173, 1956.
64. SHARP, G. S., S. LASSEN, S. SHANKMAN, J. W. HAZLET AND S. KENDIS. *J. Nutrition* 63: 155, 1957.
65. ROSENTHAL, H. L. AND J. B. ALLISON. *Agr. Food Chem.* 4: 792, 1956.
66. CALLOWAY, D. H. AND H. SPECTOR. *J. Nutrition* 56: 533, 1955.
67. CALLOWAY, D. H. AND H. SPECTOR. *J. Nutrition* 56: 545, 1955.
68. CALLOWAY, D. H. AND H. SPECTOR. *J. Nutrition* 57: 73, 1955.
69. HEGSTED, D. M. AND J. WORCESTER, *J. Nutrition* 33: 685, 1947.
70. SWIFT, R. W., G. P. BARRON, JR., K. H. FISHER, N. D. MAGRUDER, A. BLACK, J. W. BRATZLER, C. E. FRENCH, E. W. HARTSOOK, T. V. HERSHBERGER, E. KECK AND F. P. STILES. *Pa. Agr. Exper. Sta. Bull.* 618, 1957.

71. Yoshida, A., A. E. Harper and C. A. Elvehjem. *J. Nutrition* 63: 555, 1957.
72. Munro, H. N. and T. W. Wikramanayake. *J. Nutrition* 52: 99, 1954.
73. Bosshardt, D. K., W. J. Paul, K. O'Doherty and R. H. Barnes. *J. Nutrition* 36: 773, 1948.
74. Sinclair, H. M. *Old Age in the Modern World.* London: Livingstone, 1955, pp. 106–113.
75. Sinclair, H. M. In: *The Biology of Ageing*, edited by W. B. Yapp and G. H. Bourne. London: Hafner Publishing Co., 6: 101, 1957.
76. Farber, E. *Bull. Tulane Med. Fac.* 14: 71, 1955.
77. Walker, A. R. P. and U. B. Arvidsson. *J. Clin. Invest.* 33: 1358, 1954.
78. Horst, K., L. B. Mendel and F. G. Benedict. *J. Nutrition* 8: 139, 1934.
79. Nelson, W. L. *Some Chemical Aspects of Retarded Growth in the Albino Rat* (Dissertation). Ithaca: Cornell University, 1941.
80. Will, L. C. and C. M. McCay. *Arch. Biochem.* 2: 481, 1943.
81. Weinbach, E. C. and J. Garbus. *Nature* 178: 1225, 1956.
82. Shock, N. W. and M. J. Yiengst. *J. Gerontol.* 10: 31, 1955.
83. Barrows, C. H., N. W. Shock and B. F. Chow. *J. Gerontol.* 13: 20, 1958.
84. Barrows, C. H., M. J. Yiengst and N. W. Shock. *J. Gerontol.* 13: 351, 1958.
85. Knox, W. E., V. H. Auerbach and E. C. C. Lin. *Physiol. Rev.* 36: 164, 1956.
86. Litwach, G., J. N. Williams, Jr., L. Chen and C. A. Elvehjem. *J. Nutrition* 47: 299, 1952.
87. Waterlow, J. C. *Acta Unio internat. contra cancrum* 13: 704, 1957.
88. Wainio, W. W., H. Eichel, P. Person, F. L. Estes and J. B. Allison. *J. Nutrition* 49: 465, 1953.
89. Martin, G. J. Biological Antagonism. New York: Blakiston, 1951, 516 pp.
90. Ross, M. H. and J. O. Ely. *J. Franklin Inst.* 258: 63, 1954.
91. Ross, M. H. and J. O. Ely. *J. Franklin Inst.* 258: 241, 1954.
92. Ross, M. H. and J. O. Ely. *J. Nutrition* 61: 39, 1957.
93. Ross, M. H. and W. G. Batt. *J. Nutrition* 60: 137, 1956.
94. Ely, J. O. and M. H. Ross. *J. Franklin Inst.* 258: 317, 1954.
95. Ely, J. O. and M. H. Ross. *J. Franklin Inst.* 252: 277, 1951.
96. Ely, J. O. and M. H. Ross. *J. Franklin Inst.* 256: 473, 1953.

PROTEIN MALNUTRITION AND INFECTION[1]

Nevin S. Scrimshaw

Institute of Nutrition of Central America and Panama (INCAP), Guatemala, C. A.

Both protein malnutrition and acute and chronic infection are recognized to be serious and widely-prevalent health hazards, particularly in tropical and technically underdeveloped areas of the world. The extent to which each may contribute to the severity of the other receives too little attention, however, in the thinking of specialists in both human nutrition and infectious disease control.

In laboratory animals, protein deficiency induced by plasmapheresis combined with a low protein diet (1–5) or by dietary depletion alone, as discussed later in this paper, has been consistently shown to influence the course of both experimental and natural infections. Nevertheless, nutritionists in the United States and Europe have been reluctant to extrapolate these results to man because they find it difficult to conceive of comparably severe deficiencies occurring in human populations and because experiments with milder degrees of malnutrition in human subjects have frequently yielded inconclusive results. The striking experimental demonstrations that specific nutrient deficiencies may limit the spread of a virus in an animal host have also received undue emphasis, since quite the opposite is true for most other types of infections. Furthermore, secondary bacterial infections are likely to be the final cause of death in most viral diseases which end fatally.

Another major reason why so little attention is currently paid to the relationship between nutrition and infection is the failure to recognize the importance of various infections in precipitating acute clinical malnutrition. The 1955 New York Academy of Science symposium (6) discussed the topic "Nutrition and Infection" almost without mentioning the adverse effect of infection on nutritional status, yet the latter is

[1] INCAP Publication I-132.

of major importance in the epidemiology of a number of clinical deficiency diseases commonly seen in technically underdeveloped areas, including kwashiorkor (7, 8) and keratomalacia (9).

The extensive studies which have been made of kwashiorkor in children in the last decade have contributed greatly to our knowledge of human protein malnutrition. While deficiencies of calories and other nutrients may complicate the clinical and laboratory findings in this syndrome, there is no doubt that the edema, changes in color and texture of the hair, pellagroid lesions of the skin, apathy and anorexia as well as the marked lowering of serum proteins and various blood and tissue enzyme activities are largely a direct consequence of protein deficiency. These signs and symptoms respond initially to treatment with skim milk, vitamin-free casein hydrolyzates and even a suitable mixture of synthetic amino acids (10, 11) although eventually secondary vitamin deficiencies become limiting and interfere with further recovery.

It is important to remember that cases of frank kwashiorkor merely reflect the prevalence of underlying protein malnutrition in a large proportion of the child population in the post-weaning and pre-school years. In graphic terms the occurrence of kwashiorkor is like the protruding tip of an underseas mountain whose vast bulk of protein deficiency lies beneath the surface. Since kwashiorkor is known to occur in over 60 countries or territories and since a variety of infections are also a serious problem in these same localities, the relationship between protein malnutrition and infection has great importance for large areas of the world. Furthermore, research studies of kwashiorkor represent a particularly good opportunity to determine the nature and importance of these relationships.

Children developing kwashiorkor are not necessarily those receiving the poorest basic diet, but more often ones in whom some added stress has served to aggravate the underlying protein malnutrition common in the child population. There is strong epidemiological evidence to suggest that infection constitutes the most common precipitating stress factor. At the same time a large number of these children die as the consequence of an acute infection which would not ordinarily be fatal to a well-nourished child.

In Guatemala, for example, the age specific mortality rate among children 1–4 years of age is more than 40 times that for the United States. INCAP field studies have indicated that most of the difference is about equally accounted for by kwashiorkor, infectious diarrhea, and systemic infections (12). The latter are not virulent tropical diseases but the infections common to children everywhere—measles, pertussis, chickenpox, and respiratory diseases which in the United States would cause few deaths even if untreated.

The interaction of infection and malnutrition to produce more severe or prolonged disease than would result from either alone may be spoken of as synergism. The opposite type of relationship, antagonism, is confined largely, but not entirely, to specific vitamin deficiencies which in animal experiments hinder the growth of certain pathogenic viruses. In best-known examples of synergism, as used in this sense, malnutrition most probably decreases resistance (increases susceptibility) to an infection by interfering with the mechanisms of acquired rather than natural resistance. Evidence of the widespread occurrence of synergism in the relationship between malnutrition and bacterial, rickettsial, protozoan, and helminth infections as well as the predominance of antagonism in the effect of nutritional deficiency on viral multiplication has been recently reviewed in considerable detail (13). After examining the evidence for the occurrence of synergism between protein malnutrition and a variety of infections and considering some of the mechanisms involved, the authors conclude that far more clinical attention should be paid to the interrelationship between malnutrition and infection. They also emphasize that studies need to be continued in order to determine the mechanisms involved and their relative importance. This knowledge is needed for the assignment of due importance to nutritional and other control measures within public health programs in those tropical and subtropical areas in which both malnutrition and infection are serious problems.

EFFECT OF MALNUTRITION ON INFECTION

The studies demonstrating the effect of protein malnutrition on infection vary greatly in the quality of experimental design and the reliability of their conclusions (13), but leave no doubt that severe protein deficiency has a pro-

nounced effect on many infections in experimental animals. For example, protein deficiency in the mouse was synergistic with infection by *Salmonella* (14–17), *Pneumococcus* (18), *Staphylococcus*, *Mycobacterium fortuitum* and *tuberculosis* (19, 20). *Pneumococcus* in the rabbit is similarly affected (21). In the rat, protein depletion has been shown to increase the severity of infection by *Pneumococcus* (22), *S. typhimurium*, *Corynebacterium* (23), Borrelia (24) and *M. tuberculosis* (25). Work with infections of *Salmonella* (26) and *M. tuberculosis* in rats (27, 28) appear to contradict these findings but the extent of the infections was light and the conditions of these studies did not produce as severe a deficiency as in the other experiments cited. Using diets low in casein and inducing tuberculosis artificially by inhalation, Ratcliffe reported antagonism in the rat (29) and synergism in the guinea pig (30); Ratcliffe and Merrick found synergism in the hamster (31). In the mouse studies of Sengupta (32) and in the human studies of Marche and Gounelle (33), a deficiency of animal protein was synergistic with infection due to *M. tuberculosis*. In the only experimental study involving a Rickettsial infection, protein malnutrition proved synergistic with murine typhus in the rat (34).

Nutritional deficiency is much less likely to be synergistic with viral infections and is often antagonistic (13). Protein deficiency appears to have no effect on Theiler virus in the mature mouse (35, 36) nor on swine influenza in the immature mouse (37). In the mature mouse protein deficiency was initially antagonistic to swine influenza virus, but after two weeks no effect could be detected. Lysine deficiency per se does not appear to influence poliomyelitis in the mouse (38) nor does tryptophan depletion have much effect on the spread of Theiler virus in this animal (36).

It is easier to record the studies of the effect of protein malnutrition on infection than to explain the results. Numerous studies with dogs (4, 5), rabbits (3, 39), rats (1, 2) and mice (40) indicate clearly that under the conditions of severe protein deficiency, antibody formation may be adversely affected. Wohl, Reinhold and Rose (41) demonstrated that patients with disturbance of protein metabolism sufficient to cause low serum albumin show an appreciable impairment of antibody response to typhoid vaccine. Some other attempts to demonstrate this in man (42–45) have failed, presumably due to the relatively mild protein malnutrition in the subjects studied. Of course, resistance to a given disease does not depend exclusively on the anamnestic response. For example, Zucker and co-workers (46) found no relationship between the decreased ability of rats to form agglutinins to a vaccine prepared from killed culture of *Corynebacterium kutscheri* and their resistance to the live organism. The conclusion from the studies of malnutrition and antibody formations in man is not that he differs from other animals in this respect but that sufficiently severe protein deficiencies in man are not readily produced by feeding deficient diets to well-nourished individuals for relatively short periods of time, nor even by the type of depletion usually associated with chronic disease.

Another mechanism of possible importance in the synergism of malnutrition and infection is the effect of nutritional deficiency on phagocytic activity. Guggenheim and Buechler (14, 47) conclude from their work with rats that low protein diets 'invariably' interfere with leucocyte production. It is of particular significance that in these studies peanut protein promoted greater leucocyte regeneration than would be predicted from its effect on growth. This suggests once more that amino acid requirements for different body functions may differ somewhat and that a single reference protein of standard amino acid composition is not likely to be equally good for growth and for antibody formation.

Other possible mechanisms involved are discussed in the review previously mentioned (13). They include direct effect on tissue integrity, interference with non-specific protective substances, non-specific destruction of bacterial toxins, alterations of intestinal flora and changes in the endocrine balance of the host. Children with kwashiorkor provide an unusual opportunity to study the effect of severe protein depletion in man on antibody formation, leucocyte activity, and the various other functions suggested by observations in experimental animals to be of importance in the interrelation of malnutrition and infection.

EFFECT OF INFECTION ON PROTEIN MALNUTRITION

Diarrheal and other infections are exceedingly common among young children in technically underdeveloped areas. A child developing kwashiorkor usually has a history of an infection some weeks before the actual onset of the

edema, skin lesions and other clinical signs of the syndrome (48, 49). Furthermore, the majority of young children in areas in which kwashiorkor is common are suffering from a degree of protein deficiency sufficient to interfere with growth and development and often to produce changes in the color and character of the hair. It has been assumed that diarrhea of infectious origin frequently serves as a precipitating stress by interfering with the absorption of protein and thus is a major factor in the epidemiology of kwashiorkor. The effect of diarrheal infections on absorption, however, may be only a small and incidental part of the mechanism involved. Careful metabolic studies have shown that diarrhea per se does not always interfere appreciably with absorption (50). Furthermore, a variety of other infections not generally associated with diarrhea have been shown to have a pronounced effect on nitrogen excretion. These include erisipelas (51), meningitis (52), malaria (53), pneumonia, pyelonephritis, paratyphoid (54), typhoid (55, 56) and tuberculosis (56, 57).

When any of these infections are present, nitrogen excretion in the urine is invariably increased. Even if fever is present, the magnitude of the influence of nitrogen equilibrium is much greater than could be accounted for by the increase in basal metabolic rate associated with the elevation in temperature (58). It appears that the adverse influence of infection on nitrogen balance is due to actual cellular breakdown or interference with cellular metabolism rather than to either increased utilization or decreased absorption of protein.

Epidemics of kwashiorkor 6–8 wk. after an outbreak of measles have been noted on several occasions (59 and INCAP unpublished data). The degree of cellular damage would presumably be far more widely disseminated in the body in viral infections than in those of bacterial origin. The isolation by Ramos-Alvarez and Sabin (60) of a virus in 40% of children with diarrhea in Cincinnati and our failure in Central America to find a bacterial pathogen in more than 10% of diarrheas in children (59) suggests that viruses may be the most important factor in infectious diarrheas, although direct proof is still lacking.

Infection also contributes indirectly to aggravation of the protein deficiency in malnourished children. The mother, and sometimes the physician, will often withdraw from the diet of the child what few solid foods he may be getting and place him on a diet of thin cereal or corn starch gruel. The mother may also interpret the diarrhea as due to 'worms' and give the child a strong purgative. In fact, one of the important factors to consider in evaluating the effect of infection on nutrition in field studies, is the tendency of the parent to change the diet for the worse in the presence of infection.

The adverse effect of infection on nutritional status is of course not confined to children, but it is most frequently in the pre-school children that previous protein nutrition is so poor and protein reserves so inadequate that the effect is clinically detectable. Certainly some of the consequences of the multiple acute and chronic infections common among people living under unhygienic conditions are a result of their effect on malnutrition rather than the direct effect of the pathogen itself. It is important to bring diarrhea and other diseases under control as promptly as possible because of their effect on nutrition, quite apart from any toxic effects of the disease itself.

SUMMARY

There are many studies which demonstrate that severe protein deficiency may have a pronounced effect on the severity of infections in experimental animals. Nevertheless, the few studies conducted on human subjects have not succeeded in giving convincing evidence on this point, probably because they have not dealt with a sufficiency severe protein deficiency. There is considerable epidemiologic evidence, however, that infections in man aggravate protein malnutrition through their unfavorable influence on nitrogen balance. They also have an indirect effect since they diminish appetite and also frequently cause the parent or even the physician to reduce both the quantity and quality of the diet. There is a dynamic interaction between nutrition and infection which is of special importance to tropical and technically underdeveloped areas of the world where both protein malnutrition and infections are prevalent. To a considerable degree, success in improving health in such areas requires that attention be given to both the control of infections and the improvement of nutrition. Research studies of children with kwashiorkor offer a particularly good opportunity to determine the nature and importance of the relationship between infection and protein malnutrition in man.

REFERENCES

1. Benditt, E. P., R. W. Wissler, R. L. Woolridge, D. A. Rowley and C. Steffee. *Proc. Soc. Exper. Biol. & Med.* 70: 240, 1949.
2. Berry, L. J., J. Davis and T. D. Spies. *J. Lab. & Clin. Med.* 30: 685, 1945.
3. Cannon, P. R., W. E. Chase and R. W. Wissler. *J. Immunol.* 47: 133, 1943.
4. Madden, S. C. and G. H. Whipple. *Physiol. Rev.* 20: 194, 1940.
5. Miller, L. L., J. F. Ross and G. H. Whipple. *Am. J. M. Sc.* 200: 739, 1940.
6. Miner, R. W. (Editor). *Ann. New York Acad. Sc.* 63: 145, 1955.
7. Béhar, M. and N. S. Scrimshaw. *Trans. New York Acad. Med.* In press.
8. Trowell, H. C., J. N. P. Davies and R. F. A. Dean. *Kwashiorkor.* London: Arnold, 1954, p. 64.
9. Oomen, H. A. P. C. *Fed. Proc.* 17, Suppl. 2: 111, 1958.
10. Brock, J. F., J. D. L. Hansen and E. E. Howe. *Lancet* 2: 355, 1955.
11. Hansen, J. D. L., E. E. Howe and J. F. Brock. *Lancet* 2: 911, 1956.
12. Béhar, M., W. Ascoli and N. S. Scrimshaw. *World Health Organization* 19: 1093, 1958.
13. Scrimshaw, N. S., C. E. Taylor and J. E. Gordon. *Am. J. Med. Sc.* 237: 367, 1959.
14. Guggenheim, K. and E. Buechler. *J. Immunol.* 58: 133, 1948.
15. Guggenheim, K. and E. Buechler. *J. Hyg.* 45: 103, 1947.
16. Watson, M. *J. Hyg.* 37: 420, 1937.
17. Watson, M., J. Wilson and W. W. C. Topley. *J. Hyg.* 38: 424, 1938.
18. Sako, W. S. *J. Pediat.* 20: 475, 1942.
19. Dubos, R. J. and R. W. Schaedler. *J. Exper. Med.* 108: 69, 1958.
20. Schaedler, R. W. and R. J. Dubos. *J. Exper. Med.* 104: 67, 1956.
21. Wissler, R. W. *J. Infect. Dis.* 80: 250, 1947.
22. Wissler, R. W. *J. Infect. Dis.* 80: 264, 1947.
23. Miles, J. A. R. *Brit. J. Exper. Path.* 32: 285, 1951.
24. Guggenheim, K., E. Buechler and S. Halevi. *J. Infect. Dis.* 88: 105, 1951.
25. Koerner, T. A., H. R. Getz and E. R. Long. *Proc. Soc. Exper. Biol. & Med.* 71: 154, 1949.
26. Metcoff, J., D. B. Darling, M. H. Scanlon and F. J. Stare. *J. Lab. & Clin. Med.* 33: 47, 1948.
27. Lange, L. B. and N. Simmonds. *Am. Rev. Tuberc.* 7: 49, 1923.
28. Metcoff, J., D. Darling, D. Wilson, A. Lapi and F. J. Stare. *J. Lab. & Clin. Med.* 34: 335, 1949.
29. Ratcliffe, H. L. *Fed. Proc.* 10: 368, 1951.
30. Ratcliffe, H. L. *Fed. Proc.* 13: 441, 1954.
31. Ratcliffe, H. L. and J. V. Merrick. *Am. J. Path.* 33: 107, 1957.
32. Sengupta, S. R. and J. W. Howie. *Brit. J. Nutrition* 2: 313, 1948.
33. Marche, J. and H. Gounelle. *Milbank Mem. Fund Quart.* 28: 114, 1950.
34. Fitzpatrick, F. *Am. J. Pub. Health* 6: 676, 1948.
35. Kearney, E. B., W. L. Pond, and B. A. Plass. *J. Infect. Dis.* 82: 177, 1948.
36. Kearney, E. B., W. L. Pond, B. A. Plass, K. H. Maddy, C. A. Elvehjem and P. F. Clark. *J. Bact.* 55: 89, 1948.
37. Sprunt, D. H. *J. Exper. Med.* 75: 297, 1942.
38. Jones, J. H., C. Foster, W. Henle and D. Alexander. *Fed. Proc.* 6: 411, 1947.
39. Gemeroy, D. G. and A. H. Koffler. *J. Nutrition* 39: 229, 1949.
40. Toomey, J. A., W. O. Frohring and W. S. Takacs. *Yale J. Biol. & Med.* 16: 477, 1944.
41. Wohl, M. G., J. G. Reinhold and S. B. Rose. *Arch. Intern. Med.* 83: 402, 1949.
42. Balch, H. H. *J. Immunol.* 64: 397, 1950.
43. Bieler, M. M., E. E. Ecker and T. D. Spies. *J. Lab. & Clin. Med.* 32: 130, 1947.
44. Havens, W. P., Jr., D. G. Bock and I. Siegel. *Am. J. Med. Sc.* 228: 251, 1954.
45. Larson, D. L. and L. S. Tomlinson. *J. Lab. & Clin. Med.* 39: 129, 1952.
46. Zucker, T. F., L. M. Zucker and J. Seronde, Jr. *J. Nutrition* 59: 299, 1956.
47. Guggenheim, K. and E. Buechler. *J. Immunol.* 54: 349, 1946.
48. Scrimshaw, N. S., M. Béhar, F. Viteri, G. Arroyave and C. Tejada. *Am. J. Pub. Health* 47: 53, 1957.
49. Jelliffe, D. B. *Infant Nutrition in the Subtropics and Tropics.* W.H.O. Monograph Series No. 29. W.H.O., Geneva, 1955.
50. Chung, A. W. and B. Viscorova. *J. Pediat.* 33: 14, 1958.
51. Coleman, W., D. P. Barr and E. F. DuBois. *Arch. Intern. Med.* 29: 567, 1922.
52. Grossman, C. M., T. S. Sappington, B. A. Burrows, P. H. Lavretes and J. P. Peters. *J. Clin. Invest.* 24: 523, 1945.
53. Barr, D. P. and E. F. DuBois. *Arch. Intern. Med.* 21: 627, 1918.
54. Kocher, R. A. *Deutsches Arch. klin. Med.* 115: 82, 1914.
55. Coleman, W. and F. C. Gephart. *Arch. Intern. Med.* 15: 882, 1915.
56. Krauss, E. *Deutsches Arch. klin. Med.* 150: 13, 1926.
57. Rao, B. S. N. and C. Gopalan. *Indian J. M. Res.* 46: 93, 1958.
58. Peters, J. P. and D. D. Van Slyke. *Quantitative Clinical Chemistry.* Baltimore: Williams and Wilkins, 1946, p. 678.
59. DeMaeyer, D. B. Personal Communication, 1958.
60. Ramos-Alvarez, M. and A. B. Sabin. *J.A.M.A.* 167: 147, 1958.

DISCUSSION

DR. P. GYÖRGY: This symposium as those of you who have read my Introductory Remarks know, is a deviation from the customary organization of such symposia. I have to admit that this deviation is due to my own personal idiosyncrasy against long talks. I felt it was much better to distribute the papers before-hand and let you read them (most of you did not) but at least you have no excuse, and time could be reserved almost exclusively for discussion, for organized discussion. The discussion will be directed here from the Moderator's chair. Questions from the floor should be written and sent up, as soon as you've thought them out, to the head table.

Those of you who have read the title of the symposium know we will discuss "Protein Requirement and its Assessment in Man." We don't speak of 'allowances.' The term 'allowance' is misinterpreted often. It is confusing, and the figures are not necessarily based on scientific foundation but often on the available food in the area, on customs, fads or other psychological and cultural, anthropological, practices. Therefore, we are not talking about this with special reference to the dietary allowances of the Food and Nutrition Board of the National Research Council.

We have here seven speakers, and they will summarize their short or lengthy contribution in the Monograph to not longer than 5 minutes.

For systematic discussion, I will give you the headings of the Chapters which we will discuss, probably in this order: nitrogen composition of the body; maturation; protein-reserve, including liver protein, enzymes; protein requirements, biological value, net protein utilization, amino acid pattern with imbalance and unbalance; amino acid fortification; 'ideal protein,' increased intake of imbalance protein; hypoalimentation with protein, during stress, resistance, illness; and finally hyperalimentation. The discussion will be summarized by Dr. Waterlow and re-summarized by your Moderator, and the panelists will be asked whether they agree with the conclusions.

I would like to call first on Dr. Wallace. I may add that these discussions should be and will be very informal. I will ask the panelists to remain seated, even for their first 5 minutes' presentation.

DR. WALLACE: It's with some diffidence that I speak at this symposium on protein requirements. My remarks are directed towards a nutritional technique often used for assessment of protein requirements and are, in essence, critical. Furthermore, I have no better method to propose. Under such circumstances, it's probably better that one say nothing.

My interest in the interpretation of metabolic balances began some 15 years ago during experiments designed to determine changes in body composition in infants during acute nutritional episodes using balances to study retentions during recovery. We found, as did many others, that the more of a substance we fed the baby, the greater was the apparent accretion of the substance to the body per unit of body weight. This seemed to apply to all the elements in the intake and occurred in well-nourished, healthy, growing infants, as well as sick ones.

Those who study the composition of tissues have long used the protein concentrations of the tissue as a reference standard against which changes in water and electrolyte could be measured. Protein is used because of its relatively great constancy under varying conditions.

In the preprint for this symposium, I have presented certain data indicating that the nitrogen content of the body on a fat-free basis is remarkably constant despite all prior attempts to change it. The absolute size of the body may be changed by variable protein feeding, but the essential body concentration of protein appears to remain unchanged. I would parenthetically suggest at this point that there are no reserves of protein in the body in a morphologic sense; any reserve protein, it seems to me, must be thought of in dynamic-physiologic terms. How then is one to explain the inexplicable finding of a gain of nitrogen not represented by a gain of body weight?

As I have indicated, changes in body fat, body ash content, and the per cent of nitrogen in the collective proteins of the body, cannot be invoked to explain this enigma. Under certain conditions, changes may occur in the ratio of water:protein in the lean body mass, which may account for the retention of nitrogen without equivalent change in body weight.

During the early phases of growth of the human infant as much as ⅓ of the retained nitrogen may be assigned to the body without change in body weight. I've indicated that in the figure on page 1128 of the preprint. It's an interesting speculative diagram.

It's my own conviction, however, that the major explanation of the enigma lies in the problem of positive cummulative errors in the balance technique.

Inquiries of our own have shown that even with the most meticulous nursing techniques, about 2% of the intake does not enter the body, that about 2% of excreta are not recovered. In routine feeding trials, these losses can often run much higher.

These two losses (and this is the point which may seem very simple, and which I think many

people have never really thought of) do not cancel each other as is commonly supposed nor are they additive to 4%. They, when taken with the skin losses, make the balance in the case shown 15% high for the day or period of observation. In other words, very small errors come out as very large errors in the balance, and this comes about because one uses two very large numbers to get a very significant small one. If the caloric intake and the losses are kept the same and the protein intake doubled, this error would be multiplied by 2 and become 30%. When such an error is factored to assess a requirement over long periods, the result is obvious.

Before I stop talking, I should like to point out what I've not said. I've not advocated that we stop carrying out balances; when carefully done, they can tell us much about the dynamics of protein metabolism under contrasting conditions. It is primarily, I think, when the technique is applied to the assessment of requirements that the results become misleading.

Those who use the balance method should attempt to validate the data in terms of this constancy of body composition that I've spoken of. The results, when one goes through this validating process, are often very instructive.

DR. GYÖRGY: The next speaker is Dr. Hegsted.

DR. HEGSTED: As Dr. György has said, the thing we really want to know is how much protein people should eat. The accepted technique for establishing any kind of allowances (or recommendations or whatever you wish to call it) is to determine the minimum requirement, where we have something we can actually measure, and then, by some kind of gymnastics (mental or otherwise) put in an allowance for safety to give us what we think we ought to eat. I think it's clear why, in all studies, we've had to rely upon estimates of minimal requirements as the base line.

In this paper I've tried to establish, as far as theory goes, the minimal need. It's based on two suppositions or has two bases: one is that the maintenance requirement is proportional to the basal metabolic rate, and the second is that a protein with a biological value of 100 is completely utilized for maintenance and growth. I would emphasize that I think this value is minimal. I haven't pushed it as hard as I could, but I don't see how you could get estimates much lower than this line.

There have been many objections to this theory since it was published. The first one is that in the infants the values are too high. People have objected they're too high because the breast-fed infant may not get quite this so-called theoretical minimum. I've indicated in the paper that it appears that probably he can, that breast milk supplies, as near as I can tell, about the theoretical minimum. I can't find any logical basis for treating infants on a different basis than other ages, so I have to leave it as is, with whatever implications you want to draw from it, that the breast-fed infant gets just about the minimal amount of protein needed. If I lowered it for the infant of that age, then I would have to lower it across the board, and I'm not willing to do that.

I recall your attention to the fact that growth, after the first year, makes a very small contribution to the protein requirement. I think the effect of growth upon nutritional requirements in man has been overestimated.

Now, as soon as you get past infancy, then everyone's objection is that the curve is too low. I've searched for ways to raise this somehow, and I have suggested one here, namely that biological value may not be a constant. Although we are accustomed to think of biological value as determined by the amino acid composition, there is evidence that it is also determined by the nutritional status of the animal and the level of protein fed. If the biological value falls as you increase the intake, then this would give us another basis for estimating a further increase in the minimal requirement above the absolute minimum.

Figure 3, on page 1134, is about the only data I could find to try and test the hypothesis. It is of some interest that in Dr. Barnes' work on rats, when egg protein was fed, it was 100% utilized in terms of maintenance and growth, as the theory would predict, until the intake exceeded the requirement.

With the other two proteins, there is a considerable fall in efficiency of utilization, and if this is at all typical, then one might find reason here to add another 30% or so to minimal protein needs. I suggest that this is something worthy of more consideration and study.

Then, in the final part of the paper, I simply pointed out the relatively little evidence one can find of protein deficiency in adults, almost anywhere, even on diets that by most of our standards are pretty bad. Finally I pointed out that I think the problem of protein deficiency and protein requirements is a complicated one, and that I'm in partial disagreement with Dr. Scrimshaw. I don't think we do ourselves a service to oversimplify the situation or to indicate that we know more than we really do.

DR. GYÖRGY: The next speaker is Dr. Harper.

DR. HARPER: I shall confine my comments to two points: one the significance of the concept of amino acid balance in relation to protein requirement, and two, the effect of amino acid imbalance on protein requirements.

In essence, the concept of amino acid balance

involves a few, I think rather obvious, relationships. First, different proteins contain different amounts of the various indispensible amino acids, that is, their amino acid compositions are different and can be expressed as a proportion.

Second, there are differences in the requirements of the body for the different amino acids. For example, man requires four times as much lysine as tryptophan. So, these requirements can also be expressed as a proportion.

Third, if the indispensible amino acids are present in a protein, in roughly the proportions in which they are required by the body, such a protein has a high nutritive value; that is, it is a well balanced protein.

Fourth, if a protein is relatively low in one or more of the indispensible amino acids, it is of low nutritive value or is poorly balanced or unbalanced.

Finally, it requires more of an unbalanced protein than of a balanced protein to satisfy the amino acid requirements of the body. For example, if protein A contains only half as much lysine as protein B, it takes twice as much of protein A to satisfy the requirement of the body for lysine. If the lysine requirement is satisfied by feeding such a protein, then all of the other amino acids will be provided in excess and the excess can be used only as a source of calories. Therefore, the efficiency with which the nitrogen of the protein will be used for body protein synthesis will be low. Or, in other words, the protein requirement expressed in terms of such a protein will be high. Thus, the protein requirement, if we must talk about protein requirements, is determined by the amino acid composition of the dietary protein as well as by the amino acid needs of the subject.

We must also keep clearly in mind, of course, the fact that there is a quantitative as well as a qualitative aspect of amino acid balance. This is illustrated by figure 1 at the bottom of page 1137. The lower part of the figure (B) shows a comparison of the amino acid proportions of wheat and egg proteins. As you can see, if the lysine deficiency of the wheat proteins is corrected by supplementation there are only small differences in the amino acid proportions of these two proteins. (There may be some question about the figures for amino acid analyses. These are from the Summary Tables in Block and Weiss. I checked some individual analyses and you could certainly vary the proportions somewhat by selecting individual analyses for wheat proteins. Nevertheless I think the general observations hold.)

However, wheat proteins contain about ⅓ less of the indispensable amino acids than do whole-egg proteins. Therefore, as shown in the upper part of this figure (A), even when the amino acid proportions are equalized, it will still take more wheat protein than egg protein to satisfy the amino acid requirements of the body.

If the amino acid balance of a diet is sufficiently poor, the utilization of the limiting amino acid may be depressed below what would be expected on the basis of the previous discussion. This is illustrated in table 1 (page 1139).

The addition of increasing amounts of gelatin to a diet containing casein causes a gradual worsening of the amino acid balance of the diet since gelatin contains no tryptophan. As a result, not only is protein utilization poor, for example, growth is no better with 12% of unbalanced protein in the diet than with 6% of the better balanced protein, but a stage is reached at which growth is less with 18% of unbalanced protein than with 6% of balanced protein. This is an example of what we refer to as amino acid imbalance. This condition is corrected by supplementation with a small amount of the limiting amino acid in the diet, tryptophan; (the bottom row of table 1 shows that this corrects the condition).

Also, as shown in figure 2, the condition is corrected if the amount of balanced protein in the diet is raised. In this example the experimental conditions were a little different; instead of using gelatin, we used an amino acid mixture, lacking entirely in threonine to induce an imbalance, and, as you can see, when the amount of casein in the diet (casein supplemented with sulfur amino acids, so that it was relatively well balanced), was increased, the unbalanced amino acid mixture had less effect. Thus the condition was corrected by increasing the quantity of balanced protein in the diet.

It thus appears that if the balance of amino acids in a diet is sufficiently poor, there will be an increase in the amino acid requirements. Such effects become less severe the closer the dietary protein comes to meeting the entire amino acid requirements.

So, we can summarize briefly by saying that the poorer the amino acid balance of the dietary protein, the greater will be the amount of protein needed to satisfy the amino acid requirements of the body. It sounds almost like a truism when you put it that way, doesn't it? However, the amount required may be inordinately large if the amino acid balance of the diet is particularly poor.

DR. GYÖRGY: The next summary will be given by Dr. Waterlow.

DR. WATERLOW: I'm going to say straight out that I don't think enzyme studies in man are likely to give any immediate information about protein requirements, the subject of our session.

All I want to do now is to indicate some ways in which these measurements may help us in our work on human protein nutrition, and more

specifically, I can only speak about protein deficiency in infants.

In my position as a clinician, I'm presented with a patient, a baby, that is 'protein depleted,' a phrase that Dr. Wallace doesn't like, but by this I mean to indicate that the absolute amount of protein in the body is greatly reduced compared with a normal baby of the same age—and more controversial—that the relative amount of the 'mobile' protein is also reduced in relation to the 'fixed' or structural protein.

Now, I want to know three things about this patient: first, the degree of protein depletion; secondly, whether there are differences in the pattern of the depletion, as a result of differences in caloric intake, etc; thirdly, I want to know biochemical mechanisms that cause the clinical changes which we see: edema, loss of potassium, fatty liver, and so on, which we do not yet understand very well.

I think that enzyme studies can help us with the last two points, and tissue enzymes are potentially more interesting than blood enzymes or enzymes in secretions such as digestive juices.

The protein loss that occurs in these patients has been measured in two tissues, liver and muscle; but the enzyme work has mainly been done on the liver. Unless there is such a thing as storage protein, which we shall discuss, the protein that is lost from the liver must include enzymes.

Experimental work shows that on a low protein diet some liver enzymes are reduced in activity; others are unchanged, and others are even increased in relation to unit nitrogen or unit weight.

An enormous amount of animal work has been done, and no definite pattern has emerged. The same kind of thing holds in man. In the liver in kwashiorkor we find reduced activity of xanthine oxidase, d-amino-acid oxidase, and cholinesterase; no change in dehydrogenases, transaminase, or cytochrome reductase; and an increased activity of alkaline phosphatase. These are some of the results obtained by Dr. Scrimshaw and his colleagues, Dr. Mukherjee in Calcutta, and myself. There are several ways, I think, in which we can look at these results.

First, we can look upon enzymatic activity simply as a convenient label which enables us to study changes in the amount or fate of specific protein.

Secondly, we may think of the function of the enzyme, and of changes in enzyme levels as they might relate to functional damage. Is there any functional reason why one enzyme should be lost and another unchanged? There are some enzymes which seem not to be very important for life, or at least whose function we do not yet know, such as pseudo-cholinesterase. Could this be the reason why such an enzyme is lost, whereas others—e.g. cytochrome reductase—which are vital for life are selectively preserved? Admittedly, that sort of teleological statement has no meaning unless we know how the changes are brought about.

Thirdly, the change in activity or amount of an enzyme might be related to its chemical structure. An amino acid imbalance might cause loss of a particular enzyme because of the amino acid composition of that enzyme. This has been suggested in the case of the sulphydryl enzymes, when diets are low in sulphur-containing amino acids, but again there isn't any evidence that this actually happens.

Lastly, there is the structural pattern in the cell. A change in enzyme activity may be due not to a change in amount of an enzyme, but to a change in spatial relationships.

I think if we pursue these lines, we may throw some light upon two of the questions which have been raised: the question of differences in pattern of protein depletion, and the nature of the cellular mechanisms and changes that occur.

I feel that the simple clinical approach, of looking for changes in one enzyme in one tissue at one moment of time, will never be of much practical value for the assessment or the prognosis of protein deficiency in man.

DR. GYÖRGY: Thank you. According to the program, Dr. Scrimshaw should follow Dr. Waterlow.

DR. SCRIMSHAW: Both protein malnutrition and acute and chronic infection are serious and widely prevalent health hazards in tropical and technically underdeveloped areas of the world. Nevertheless, I believe that the extent to which each may contribute to the severity of the other in such areas receives too little attention in the thinking of specialists in both human nutrition and infectious disease control.

For example, there is a general failure to recognize the importance of various infections in precipitating acute clinical malnutrition. In a 1955 New York Academy of Science Symposium on the topic, 'Nutrition and Infection', the discussion neglected almost entirely to mention the adverse effect of infection on nutritional states, yet this effect is of major importance in the epidemiology of a number of clinical deficiency diseases seen in technically underdeveloped areas, including kwashiorkor and keratomalacia.

In appraising the public health significance of kwashiorkor, it is important to remember that frank cases merely reflect the prevalence of underlying protein malnutrition in a large proportion of the child population in the postweaning and preschool years. Since a variety of infections are also a serious problem in these same localities, any interrelationship between protein malnu-

trition and infection obviously has importance for large areas of the world.

Children developing kwashiorkor are not necessarily those receiving the poorest basic diet, but more often are the ones in whom some added stress has served to aggravate the underlying protein malnutrition which is common in almost the entire child population. There is strong evidence to suggest that infection constitutes the most common precipitating stress factor in kwashiorkor. Furthermore a large number of children, with underlying protein malnutrition, die as the consequence of an acute infection which would not ordinarily be fatal to a well-nourished child.

In Guatemala, for example, mortality rate among children 1–4 years of age is more than 40 times that for the United States. INCAP field studies have indicated that most of the difference is about equally accounted for by kwashiorkor, infectious diarrhea, and systemic infections. The latter are not virulent tropical diseases but the infections common to children everywhere: measles, pertussis, chickenpox and respiratory infections, which, in the United States, would cause few deaths even if untreated.

This interaction of infection and malnutrition to produce more severe or prolonged disease than would result from either alone may be spoken of as synergism. The opposite type of relationship, antagonism, is confined largely, but not entirely, to specific vitamin deficiencies which in animal experiments hinder the growth of certain pathogenic viruses. It should be made clear that malnutrition may have little or no effect on the incidence of infection, but the synergistic combination of malnutrition and infection may greatly increase morbidity, and even mortality.

In an article published in the March issue of the 'American Journal of The Medical Sciences', Carl Taylor, John Gordon and I, have reviewed a large number of references which point to evidence of the wide-spread occurrence of synergism in the relationship between malnutrition and bacterial, rickettsial, protozoan, and helminth infections as well as the predominance of antagonism in the effect of nutritional deficiency on viral multiplication.

Unfortunately, much of the work is poorly controlled and there are almost no good epidemiological studies among malnourished human populations. Further knowledge is needed to evaluate the net effect of infection on protein requirements, and conversely, the extent to which the presence of malnutrition increases the duration and severity of the illness resulting from infection. These interrelationships are of great importance particularly in those tropical and subtropical areas in which both malnutrition and infection are serious problems.

DR. GYÖRGY: Thank you. Dr. Watkin?

DR. WATKIN: When you consider the difficulty that the other panelists have had in assigning a protein requirement to the normal man, the normal animal, or to one segment of the human race, notably infants, it is not surprising that when Dr. Levenson and I were confronted with the problem of the protein requirements and their assessment in the injured and sick, we were somewhat staggered by the job.

When a physician is confronted with an injured or sick individual, he must, of necessity, make a decision with regard to the protein nutrition of his patient. If he decides on one course, he may be gratified by a satisfactory recovery of the patient from his affliction; if he chooses another, he may witness a succession of complications, prolongation of morbidity, and not uncommonly, in certain situations, the death of the patient. The decision, therefore, is not one to be subrogated in importance to other surgical and medical considerations. However, if this physician is seeking a solid rule of thumb to lighten the burden of his decision, he will not find it in the paper prepared as a basis for this symposium and probably not in the discussions this afternoon.

The major points that Dr. Levenson and I wish to emphasize are the individuality of patient response to seemingly similar injuries and illnesses and that the nutritional care of the patient is not an entity unto itself but is rather an integral part of the total care of the patient. A correlated point deserving emphasis is that proper regard for the nutritional problems of the patient is critical and essential for optimum medical and surgical care.

Certain guide lines will assist the physician in reaching his decision. Many patients who are injured or have certain acute and chronic diseases demonstrate a net loss of protein from their bodies. At first glance, it would seem that replacement is desirable. And, for most situations this is clearly so. However, the rate at which repletion is undertaken must be adjusted to the particular clinical situation for there are some patients for whom attempts at rapid, heroic repletion is undesirable and even dangerous. Generally, the previously healthy patient suffering from acute injuries, acute infections, or hyperthyroidism may be repleted fairly rapidly with benefit provided the period of malnutrition has not been prolonged. If the latter situation is the case, nutritional repletion must be undertaken more slowly. Similarly patients with certain neoplastic diseases, certain types of chronic liver disease and certain chronic infections may fare better if their dietary intake, oral and parenteral, is increased rapidly. But, sweeping generalizations are misleading. Patients with neoplasia must be divided into those with inactive and active malignancies. Patients

with chronic liver disease must also be divided into those with active and inactive varieties. Patients with chronic infectious diseases must be considered in the light of the infecting agent, the resistance and response of the host. Even when these factors are considered, the guide lines are not very clear. The physician must consider also the role of all nutrients, not just protein, the effects of medication and drugs, such as aspirin and digitalis, plasma extenders, antibiotics, tranquilizers, anesthetics, operations and even such procedures as induced hypothermias. The physician must estimate somehow the severity and significance of the total insult the patient has suffered.

Their aim should be to provide the means whereby the patient can recover optimally from his injury or illness. When we are confronted with a problem such as the malnourished patient with severe chronic dysfunction who needs protein but for whom protein in high dosage may be dangerous, we must seek better means than are now at hand. The answer must come from basic nutritional biochemistry, from experimental surgery, radio-biology, or antibiotic development.

In conclusion, the assessment of protein requirement for the injured or sick man must be made for each individual with due consideration to all aspects of his particular plight as well as to the best interpretation of currently available evidence on the optimum requirement for protein and other nutrients.

DR. GYÖRGY: Dr. Ross, you are the last speaker to summarize your contribution.

DR. ROSS: As a result of our earlier experiments on the influence of different diets on a number of biochemical entities, a large scale qualitative study was begun several years ago to determine the effects upon degenerative diseases and life expectancy in rats fed certain rations for their entire lives.

The 4 semi-synthetic diets used were designed so as to permit similar or different allotments of proteins, carbohydrates, or of calories. The intake of other constituents in each diet were adequate and kept constant. A fifth group received commercial diet.

Although these long-term studies are not yet completed, the data permit certain conclusions to be drawn.

The shortest average life span and the high incidence of degenerative diseases were found in rats maintained on commercial diet. These results are of interest for purposes of comparison with those of other workers, but because of the many unknown aspects of this diet, results are of limited value in a comparative analysis of experimental diets.

Since it appears that some of the diets used in these studies have different influences at different ages, a single value representing average life expectancy in these instances, may not be indicative of the effect of these diets. For example, one group whose life-time intake of protein, carbohydrates and calories was low, suffered a higher mortality rate early in life than another group of rats whose life-time intakes of these dietary constituents were more than three times as great; at later ages the pattern was reversed, and the animals with a life-time of low intakes had the lower mortality rate.

No really pronounced modification of life expectancy could be shown after a life-time of protein intakes differing by more than threefold in two groups of rats receiving identical amounts of isocaloric diets.

Although expectation of life after 2 years of age in the rat appears to be correlated with caloric intake, the modifying influence of the protein level upon age associated degenerative diseases seemed to be more definitive. A high caloric intake was found to be related to a high incidence of spontaneous tumors but when given in conjunction with a high protein intake, there was a doubling in tumor incidence. For example: in two groups of rats consuming identical amounts of isocaloric diets but in which the allotment of protein to one group was more than three times that of the other, there was a 40% tumor incidence in those rats still alive after one year of age on this high protein allotment as compared to the 20% incidence with the lowered protein allotment. A reduction in total caloric intake still further reduced the incidence but when given in conjunction with a low protein intake, the spontaneous tumor incidence was reduced to a value of only 2%.

Further evidence of the effect of increased levels of dietary protein in isocaloric regimens are seen in an increased incidence of cardiac, renal, and general vascular disease. These data are based on gross pathological observations and have been, in part, confirmed by microscopic studies.

These data appear to relate to growth rate and give us the impression that early nutritional states may be a determinant in the incidence and severity of certain age-associated diseases.

It is not yet possible to designate, from these studies, the ration that will result in a further enhancement of life expectancy at any age, as well as the reduction in the incidence of degenerative diseases.

It would appear that a diet containing 30% protein and allotted in the amounts used in these studies is too high and, for the rat, a diet containing an 8% level of protein may be too low.

New lifetime studies to aid in determining the

optimal levels of protein, carbohydrate, and caloric intake are now being designed.

DR. GYÖRGY: Thank you very much.

In the following general discussion the Moderator will direct his question to one of the panelists, but all panelists have not only the right, but they are invited to enter the discussion.

According to organized discussion, I would like to start with static biochemical problems in contrast to dynamic biochemistry. Therefore, we have to quiz and squeeze Dr. Wallace first because he was discussing the nitrogen content of the body. The first question is, whether the body composition, the total body nitrogen or protein composition, is a fixed one. I would like to ask Dr. Wallace, are there perhaps, age determined variations in total body-protein?

DR. WALLACE: Yes, I think there are. When the baby grows from the newborn period or from the premature period, he changes the ratio of water to protein in his body (his fat free body as well as in his whole body). The protein content rises from about 2% nitrogen in the normal infant to about 3% in the adult. This process is pretty well accomplished by the time a child is a year of age. This iss esentially a rearrangement of the ratio of water:protein in the body and is an event which occurs in all animals and seems to occur at about the same rate in relation to the total life span although that problem is still controversial.

Whether one can speed this up or not is a question now asked by many people who are interested in the feeding of infants. I personally don't think that one can speed up this biologic process but it can account for a considerable gain of nitrogen around 3, 4 and 5 months of age where as much as 30% of the nitrogen retained can be assigned to change in the water:protein ratio.

At the same time, there are changes in the amount of muscle, of bone, connective tissue and so forth, relative one to the other as this process goes on.

DR. GYÖRGY: I have more questions. Do you think that the whole of the apparently greater nitrogen retention by babies on a high protein level can be explained by technical error; in other words, do you think that chemical maturation of the soft tissues is never speeded up; if it is speeded up, is this of any benefit to the patient?

DR. WALLACE: As I've indicated, I don't think you can speed this up, I am not certain, but from all the evidence I know from animal work and also indirect evidence we have from human work, there seems to be no evidence that one can speed this process up by giving more protein.

DR. WATERLOW: I'd like to interpose, if I may, that the malnourished baby, even in the absence of edema, is a little like the very young baby in that it still has a higher water content and a low protein content, and you can certainly speed up the rate at which this is restored to normal by the amount of protein you feed.

DR. WALLACE: Now, where is that water? Is it outside the cells or inside the cells?

DR. WATERLOW: Unfortunately, I don't think anybody really knows. The experimental evidence on this as far as I know is not conclusive.

DR. WALLACE: During maturation of the growing infant, most of that change in water takes place in the so-called extracellular fluid.

DR. WATERLOW: In protein deficient infants a great deal of this extra water, even without edema, is extracellular but some people think that some of it is intracellular.

DR. GYÖRGY: Maturation coincides with the highest rate of growth?

DR. WALLACE: Yes, at the period of maximum growth and also at the time of maturation of all sorts of biochemical and physiological functions.

DR. GYÖRGY: You would feel under normal conditions, meaning normal dietary conditions, you would have a constant concentration of body nitrogen?

DR. WALLACE: At any given age, yes. One could draw a statistically significant curve to describe it. This would help you only for a group of infants. It wouldn't help you for any individual person, of course.

DR. GYÖRGY: How could you, in a young child, an infant (a living one), determine the body composition, say by helium displacement or by other modern methods?

DR. WALLACE: One can measure body density you know. It can be much better done in adults. To try to do that in infants is a task that I've never had the courage to try and do.

One can get at body composition by helium displacement, by isotope dilution, and so forth. All of these methods theoretically could be used. Here again, you get into difficulty because all of those methods have an error attached to them that's uncomfortable. Total body water can be measured by plus or minus 5% if one's technique is very good. Also, variation here is largely a measure of fat content. So, I don't think even the dilution methods and similar techniques will give us the refinement needed to get the answer that you want.

DR. GYÖRGY: I don't want any answer. I want the truth.

DR. WALLACE: I think we will have to find other means than these techniques to get the answer.

DR. GYÖRGY: Is the relatively constant concentration of nitrogen in the fat free body limited only to the entire body or does it hold for individual organs as well?

DR. WALLACE: I think that certainly it seems

to hold for individual tissues like skin, muscle and liver within the limits dictated by the maturation process.

DR. GYÖRGY: But only under normal dietary conditions, or would you . . . .

DR. WALLACE: Even under abnormal dietary conditions, I do not think that much change can occur except for relative change in organ size.

DR. ROSS: In respect to the nitrogen percent of an individual organ: we have noted that significant differences do exist in the liver of the rat according to the nutritional regimen. For any one diet there is a relatively constant level of nitrogen in the liver regardless of age beyond the first 100 days. The level of hepatic nitrogen correlates directly with the proportion of protein in the food and appears not to be influenced by the quantity of food protein consumed. These conclusions hold regardless of whether the basis for calculations is wet weight, dry weight, or fat free.

DR. GYÖRGY: Dr. Waterlow, do you have any comments on this problem?

DR. WATERLOW: I think that the total nitrogen content or ratio of nitrogen:water, probably remains unchanged; but the proportion of different proteins and other constituents does not. You see this very clearly if you relate protein or nitrogen content to DNA as the most constant, although by no means completely constant, cellular constituent. Then one finds that the ratio of nitrogen to DNA in muscle and in liver, both in the rat and in the human subject is reduced to ⅔ or ½ of normal.

DR. GYÖRGY: Well, it seems to me that this is a very important change in the protein composition of the tissues, which just has not shown up by a single method of analysis, and therefore Dr. Wallace is oversimplifying the situation in a way.

DR. WALLACE: Certainly I'd agree. One of the changes we found in analyzing whole tissues and whole bodies for example, is that one can change the amount of collagen to non-collagen protein in the body.

I'd like to emphasize again that what I'm talking about is the interpretation that one gives to the nitrogen balance. Where do we assign the nitrogen gained?

As Dr. Hegsted once pointed out, and I think a very good way of putting it, a balance is very much like looking only at the interest in your bank book. This doesn't always tell you whether the bank is stealing your capital or how much capital you've got, what kind of stocks, mortgages, first or second, that your money is invested in. It only tells you how much interest they're giving you.

DR. GYÖRGY: There was a question here about the term, 'protein depletion' and I understand you are not favoring the expression. Could you tell us why not?

DR. WALLACE: To me a protein depleted child is one who is simply smaller than something which I do not understand requires him to be. Why and how one does best at a certain required size is to me the crucial question. A protein depleted person is simply smaller than he ought to be.

DR. GYÖRGY: And how about the adult?

DR. WALLACE: He must be smaller also.

DR. GYÖRGY: If he became depleted after his growth was completed?

DR. WALLACE: He would lose weight.

DR. WATERLOW: But he's not just smaller. His make up is abnormal. I mean he's got more skin and relatively more blood; he may have less of other tissues but his brain is normal in size and his kidneys are not too bad.

DR. GYÖRGY: Has he lost the most important part of his body?

DR. WATERLOW: What I mean is that he has not just uniformly shrunk, like a deflated balloon.

DR. SCRIMSHAW: There is a dynamic as well as a static aspect; if a protein depleted child is given a certain amount of dietary protein, he may retain 40 or, even under certain circumstances 50%, and a child who is not depleted will not retain nearly as much. Even if he is actively growing he will retain at the most 8–10%. They are in two different physiological states.

DR. WALLACE: I would agree with Dr. Scrimshaw that the percent of retention in a depleted individual should decrease with repletion. Allison has emphasized this. However, much data in the literature do not show this slackening in the positive balance. In some data I examined recently, backward extrapolation of the nitrogen retention data indicated that the initial total body-protein must have been 2% of the whole. This degree of depletion is difficult to accept and, to formulate, as was done; a protein requirement from such data would seem to be absurd. Balance data is very expensive data to obtain. If it cannot be validated in terms of reasonable body composition the expense would seem wasted. We need a more imaginative way to get at the problem. I am not imaginative enough to see how.

DR. GYÖRGY: You were imaginative enough to start it.

DR. SCRIMSHAW: Well, it depends upon the use that you make of balance data. To use them to calculate the amount of protein required to make up the deficit is not justified, but as an indication of the relative depletion of the organism, they are very useful.

DR. WALLACE: Cannot one do it more simply weighing the individual?

DR. GYÖRGY: Well, we are turning from the traditional concept of nitrogen values, analytical

values, to such concepts as protein reserve, labile protein. Who would like to discuss it? Do you have anything to say about protein reserve again, Bill?

DR. WALLACE: No, I've just said that I think that it is morphologically there. I may be wrong.

DR. GYÖRGY: It is stated all over, even in the last Monograph of the Food and Agriculture Organization on 'Protein Requirement', that for safety we have to add to the minimal protein requirement 25% in order to build up 'protein reserve'.

I think it's perhaps a cause for arguing with Dr. Hegsted who said that the breast-fed infant must have minimal nitrogen or protein intake. I think his must be the optimum one.

What is meant by this elusive term 'protein reserve', a reserve which we have to build up or maintain by increasing the protein intake 25% daily? If I should take 25% more calories than necessary, I will get fat, and that would not do.

DR. HEGSTED: It has been shown that animals that are fed on high protein diets certainly are different. If you take an animal that's accustomed to a low protein diet and put him on zero intake, he loses very little, whereas an animal fed on a high protein diet will lose a lot. In other words, there are some body-proteins that can be identified as more labile than others.

DR. GYÖRGY: Dr. Harper?

DR. HARPER: What are these labile proteins? They're not morphological entities. I'm not sure that I am satisfied with this terminology. Fat stores can be measured but when one starves a rat or a man, he first loses protein from his liver. Then his liver tends to stabilize and he loses protein from skin and skeletal muscles. As we've heard, his brain, kidneys and certain other organs are relatively little affected.

DR. WALLACE: Well, when he does that he makes the total amount of muscle, skin, or bone in his body smaller. He seems to maintain the concentration of protein. He doesn't come out a 'diluted' individual. Certain organs become smaller in a certain priority.

DR. ROSS: Do you consider this shrinkage as evidence of a labile protein reserve? After all if glycogen is removed in large quantities, the liver also shrinks in size; if protein is removed, then again it shrinks in size. There may be a little difference in the distribution of these two components, but can you consider this as a labile protein reserve?

DR. WALLACE: Yes, I'd certainly agree that liver represents a labile protein reserve; but a relatively small one. I tried to emphasize that I think body composition is important and nutrition is the way to study it. I often argued with Dr. György about this, and he said, "No, it's the other way around".

So you have to—I don't know, I don't want to force my point of view on it, but I'm looking at it from the standpoint of chemical anatomy, chemical morphology. I think it's there and can be used in certain priorities and how the priorities are arranged are more interesting than the fact it is used up. How does the body arrange these priorities on different tissues?

DR. WATKIN: Dr. Wallace, you're implying that the loss of protein would be accompanied by the death of a cell, would it not? Is there an actual decrease in the number of cells present in a given tissue?

DR. WALLACE: That's an interesting point. Can a cell lose, say, one half of its protein and still live? In protein depletion do one half of the cells disappear and the remainder have a normal composition? Or are all the cells smaller?

DR. SCRIMSHAW: Part of the protein of a cell can go out without the cell dying.

DR. WATERLOW: It certainly gets smaller.

DR. WATKIN: I can see that a change in concentration of electrolytes in a cell, could occur without causing the death of a cell, and without demanding that this cell may change in its absolute volume. Why can't you assume the same thing for protein?

DR. WALLACE: When potassium is lost from a cell it is nearly all replaced by sodium. The net electrolyte content remains the same.

DR. WATKIN: The osmolarity of body fluid may vary considerably in disease but at any given time is almost uniform in all fluids in the body. This would not be true if cells changed only their volume and not their internal osmolarity.

DR. WALLACE: Well, I think most people would say that the cell cannot tolerate much change in osmolarity. Now that is, of course, a controversial subject but most people today, I think, have come back to the idea that cells and extracellular fluid are in osmotic equilibrium, and they cannot greatly change their osmolarity without death of the cell occurring.

DR. HEGSTED: It is true that the liver can change in protein deficiency—it looks depleted on histologic examination. Does the protein concentration change?

DR. WALLACE: No, I don't think there's any evidence that you can change the fat-free, glycogen-free protein concentration in the liver. I think Dr. Ross could perhaps speak about this.

DR. ROSS: The activity of a large number of hepatic enzymes has been shown to be alterable by changes in the nutritional state of the rat. If the amount of activity represents changes in amount of these specific protein entities then we can demonstrate that some of these protein mole-

cules are so labile that their levels can be increased or decreased drastically by nutritional changes. Similar but slower changes occur during normal ageing. Rather than a fixed value we should think in terms of ever changing values within wide limits. I think this is part of the story of not protein reserve but the ability of protein in cells to compensate for loss.

DR. GYÖRGY: Well, I would like to ask a question of anybody on the panel; anybody on the panel can answer it or try to answer it. A true protein reserve should serve as an emergency ration. A person who suddenly has insufficient amount of protein in the diet should be able to call on his protein reserve. Is such a concept correct? Or are the protein reserves mainly enzymes which in case of undernutrition, including protein deficiency, are not needed, are catabolized and their N excreted? At least, to a considerable extent.

DR. SCRIMSHAW: Well, are not we using the term protein reserves to cover three different stores. In the broad sense, the breakdown of muscle tissue in starvation furnishes a protein-reserve. The loss of protein from the liver, presumably without a decrease in the number of liver cells, is a second type. A third type would be part of the protein in plasma and extracellular fluid.

DR. GYÖRGY: What is the concensus of the panel with regard to protein reserves?

DR. HEGSTED: I still vote for protein reserves. (laughter)

VOICE: I vote for it too.

DR. WATKIN: I think our problem, Dr. György, is in the name of 'protein reserve', and I think it may be more recognizable if described in other terms. The designation 'protein reserve' may indicate expendable cells, such as those in the liver which may die to furnish protein but which may later be pushed out of the living cell, either by the shrinkage of the cell or a change in the concentration of the protein within the cell.

DR. GYÖRGY: I think we'll come back to this problem when we discuss protein requirement, especially later when we discuss infection and stress; also the question of whether these protein reserves should express themselves in modification of the protein requirements, or whether we need more protein to keep the protein reserves high.

I would like to come to the salient point: can we determine protein requirement and what are the figures? Dr. Hegsted calculated these figures and you read his paper. There could be perhaps some objection made on the basis alone of the calculation.

In a recent paper in the Journal of Pediatrics (54: 515, 1959) Gordon and Ganzon write: "The allowance for 'maintenance' protein 12.5 mg of protein per basal calorie, is estimated from the urinary nitrogen excretion of animals of different species on intakes of no or minimal amounts of nitrogen." No allowance is made by the author for increases in urinary nitrogen, stemming from the protein ingested to cover the basal calories or the growth allowance which during the first half year is larger than, or as large as, that calculated for the basal calories, nor for dermal excretion. What do you say about this objection?

DR. HEGSTED: Well, I think he's really objecting to or refuting the idea of biological value because by definition biological value of 100 means 100% utilization; therefore, if you increase the intake of a protein with a biological value of 100, you do not increase the urinary losses—there are no losses associated with it, and from the data that I showed from Barnes' paper on rats, you can see that with egg protein, up to the level of maximum weight gain, the theory accounts for all of the protein. I just don't agree with this statement.

DR. GYÖRGY: Therefore, you feel the calculation is a good foundation for setting up figures for minimal requirements?

DR. HEGSTED: I think it's the only theoretical basis we have and, unless there are some other basic considerations, then the minimum requirements have to agree with the theory, or any discrepancy has to be explained on some logical basis.

DR. GYÖRGY: In your calculations for children, infants, rightly you referred only to the high protein-containing food, the protein food with high biological value such as milk; and for adults you made some correction of your previous figures and you increased the protein requirement from 0.35 gm–0.65 gm per kg/day. Would you comment on that?

DR. HEGSTED: I think the data just aren't available to determine how much biological value decreases as you approach optimum intakes. I think it does but I don't believe we have enough data to really define it quantitatively. At least I see a way to get my estimates a little higher and still preserve my so called 'scientific approach'.

DR. SCRIMSHAW: This is a basic point that was stressed in the discussions of the 1957 FAO Committee on protein requirements and emphasized in their report; no set of requirements, based on studies of well nourished persons in the developed countries, can apply to all socio-economic groups all varieties of diets. This is just axiomatic; you cannot even assess requirements by countries because they vary with the habits of each socio-economic group, especially with the quantity and the quality of protein consumed. I mention quantity as well as quality because apparently the level of protein intake does have effect on the biological value. The child-feeding studies with

various levels of vegetable protein, carried out by E. M. DeMaeyer in the Belgian Congo indicate this particularly well. It must be recognized that whatever requirements are worked out or agreed upon for the population of this country, will have to be reexamined in reference to the diets of persons in other areas. It is more complicated than this, however. Protein requirements are influenced not only by the balance of amino acids, but also by the frequency of infection, and allowances have to be made for the incidence of infection in individual population groups.

DR. HARPER: In other words, there is no such thing as a single protein requirement?

DR. SCRIMSHAW: Exactly.

DR. HARPER: And I think this probably should be emphasized much more than it is. In the first place, there's no evidence that man requires protein as such, is there? He requires a few specific amino acids, a certain amount of nitrogen and some carbon skeletons. Even the specific requirements for amino acids are variable, but they are certainly much more meaningful than a protein requirement, which is influenced by the composition and the digestibility of the protein. Also, depending upon how the protein requirement is reported it may be influenced by the type of diet.

DR. SCRIMSHAW: I think we should do more research on the reasons for the variability in protein requirements.

DR. HARPER: When we turn to practical nutrition, I do feel that the best approach to the problem of protein and amino acid requirements, the most sound one that I can think of, is to take a protein with a biological value of 100 and determine the minimum quantity that is required under basal conditions. Then with this as a base, requirements under other physiological or pathological conditions can be determined.

DR. GYÖRGY: It is even simpler to take as a model the breast-fed infant, at least for the first 6 months of life, when we know everything, or almost everything. We know the caloric requirement which has long been well established (100–120 cal per kg/day). We know how much protein is in the amount of human milk, which would correspond to the daily caloric requirement. This would lead to a figure for daily protein-requirement of 1.7–2.0 gm per kg. I am aware of some opposition to this teleologic reasoning. However, I am too simple to accept any such type of opposition. I just don't see its practical validity, knowing the century-old results with breast-fed infants.

DR. HEGSTED: But there you get into biological values you know nothing about.

DR. GYÖRGY: Well, fortunately, you see if my advice were followed, then the infants, the young infants, would not get protein of low biological value.

DR. SCRIMSHAW: There are complications though when the mother gets sick or dies.

DR. GYÖRGY: Well, then you get the Grandmother, in Guatemala they do it, or a neighbor, or get the cow. Now we come, of course, to a rather difficult problem. The amino acid pattern has been used as a basis of calculation, in the last report from FAO on dietary requirements, for protein requirements. Now, before we come to the so-called ideal protein, who would like to start the argument? Who's for it, who's against it?

DR. SCRIMSHAW: I'll start the defense of the concept of a reference amino acid pattern as part of a theoretical reference protein. If an actual food protein is taken as a reference there are other elements in this food beside the amino acids which may influence the utilization of the amino acids and there are also excesses of some amino acids over and above requirements which again can influence protein utilization.

In theory there ought to be an optimum dietary level for each of the essential amino acids plus a proportion of non essential amino acid nitrogen which would give us a reference point free from the extraneous influences.

Of course, the present FAO reference protein with its theoretical amino acid pattern is only a rough, first approximation, in retrospect perhaps not a very good approximation, but it has stimulated a great deal of research. Among other things, this research has indicated the difficulties in using a single protein-containing food for reference purposes. Take the specific example of corn: we have supplemented corn protein to the level called for by the amino acid pattern of the FAO reference protein and when methionine is added in this amount, the result is adverse. Nitrogen retention is depressed. This can be ameliorated by the addition of isoleucine, but not entirely corrected. Clearly something in the remaining amino acid pattern or in the absorption of amino acid nitrogen from corn is influencing the methionine requirement and making it critical because the same amount of methionine in a wheat based diet, does not have this adverse effect.

Theoretically we know a lot about eggs and about milk, but we do not know the extent to which their amino acid excesses or other factors may be influencing the effect of their amino acid pattern.

DR. GYÖRGY: In a protein food of high biological value, do you expect any major influence of any so-called imbalance?

DR. HARPER: I think it's pretty obvious that even with proteins of low biological value it depends entirely upon the level at which the protein is fed. And more important, without

amino acid supplementation, it is very difficult to demonstrate the occurrence of an amino acid imbalance.

In our animal experiments, if we use a protein such as wheat gluten (which is already so severely out of balance that one might think in terms of it having a built-in amino acid imbalance) as the sole source of dietary amino acids, we can demonstrate that the lysine requirement for maximum growth is higher than the accepted value. This is the only example I can suggest of an imbalance demonstrated with a single dietary protein without the use of supplementation procedures, and this was with a severely unbalanced protein.

DR. GYÖRGY: I am basically against the so-called ideal protein pattern because, especially in their practical sense, for a publication of FAO, I think it is sort of out of place. It has no great significance for practical nutritional studies.

DR. HARPER: You think there's no value to it?

DR. GYÖRGY: In addition, I'm humble enough not to try to improve very much on nature, and I am satisfied with the breast milk for the human infant. I don't want to improve on it. You see that's my idiosyncrasy, and I am satisfied with cow's milk and meat and other protein as substitutes, for older infants and children. The biological value of these protein foods is high enough that, for the range in which we will use them, there will be no effect of any imbalance.

DR. HARPER: I don't think I could argue with you in terms of practical application, but let's take a look at Dr. Hegsted's attempt to estimate minimum protein requirements. I think we ought to take this attitude for the sake of fundamental scientific studies and attempt to determine, if we can, whether there is such a thing as an ideal amino acid pattern for use under certain conditions. This is my reason for supporting Dr. Scrimshaw's point of view. I feel this is a fundamental approach which may eventually serve as a base for the determination of minimum nitrogen and amino acid requirements.

DR. SCRIMSHAW: Do you know positively that there is not something else in breast milk besides the amino acids that will influence the requirements for essential amino acids?

DR. GYÖRGY: I would object to the statement as I don't think it's right to talk only of amino acids. We are still eating fairly good food and not amino acids. I am only concerned about the food which we would eat and what we would do, for instance, in a country, to the population (children, adults) with the food which they grow. At present I think you have to study, and you do it too, every food mixture that you want to give to children or adults, and you have to study it as such.

DR. SCRIMSHAW: The reference pattern is the basis for such studies.

DR. GYÖRGY: And for that reason, I say study only food as it exists.

DR. SCRIMSHAW: But to what are you going to refer this? Now, you say refer it to eggs or refer it to milk.

DR. GYÖRGY: Because you can control it.

DR. SCRIMSHAW: You may refer it to something which doesn't have amino acid excesses or deficiencies which may interfere with the utilization of the one we want to test?

DR. WATERLOW: What is the point of a base line which is changing all the time?

DR. GYÖRGY: Well, I agree with you it is confusing.

DR. SCRIMSHAW: There are two points. First, should we continue efforts to make successive approximations to a reference amino acid pattern? I feel that we should continue to make these approximations. Secondly, should we refer, in the meantime, to what is admittedly an approximation, or should we, for convenience, continue to refer to egg or milk? It really does not make much difference, as long as you specify your reference basis, but it does help towards getting a better approximation, if you try to use that of the FAO reference protein.

DR. HARPER: We don't eat crystalline vitamins but we don't give vitamin requirements as so much yeast per day, or so much cabbage, or something of this sort.

DR. GYÖRGY: But, even if you had pure proteins, you would have proteins of different biological values.

DR. HARPER: Still, you like to have the best approximation of the requirements as the base for your recommendations.

DR. GYÖRGY: Now what does the panel say about the protein requirement and the various proteins of different biological value? I may also ask a further question, since this question has been sent to me by one of the experts on protein metabolism here in the audience. He asks if one could not use the breaking point in biological value with increasing concentration of the same protein in the food for determining the requirements for this particular protein-containing food. For instance, at the 10% level in the diet, as originally postulated, egg albumen has a biological value of 100. At higher concentration, around 30, there is a sudden break and reduction in the biological value, with simultaneous excessive catabolism of the protein. The question is: could this technique not be used as an experimental tool for determining protein requirements for various protein containing foods?

DR. HARPER: I think it depends on how sharply the curve that Dr. Hegsted published, breaks.

There is a very sharp break for whole-egg protein which indicates that you could come within a very narrow range in selecting the breaking-point in this case, but if you look at the other 2 curves, I don't see a break.

DR. GYÖRGY: It didn't go higher?

DR. HARPER: It didn't go high enough.

DR. GYÖRGY: Well, there's always a break. Professor Platt has now gone into quite a number of them, and he always found a break, which might vary between 30–40% of the protein in the diet, or even higher. The break might be flat, indicating a range and not a sharp point. It is perhaps preferable to speak of a range and not of one fixed figure for the biological value of proteins. Anybody disagree with a range?

VOICE: We wouldn't dare.

DR. SCRIMSHAW: One difficulty with evaluating some of the proteins and even some of the diets, is that the protein quality is so poor that you cannot eat enough to get to the breaking point.

DR. GYÖRGY: That's true, but fortunately that wouldn't be very good for people either.

DR. SCRIMSHAW: This is a point that a good many in the audience may not realize. When you are working with children, you are limited to feeding proteins which you can get in a fairly concentrated form; you cannot give the 2–3 gm of protein per kg required by young children in the form of corn or wheat or other cereals unless you use some way of fortifying them with protein.

DR. GYÖRGY: Could amino acids replace protein and how about the higher caloric requirements to utilize them?

DR. HARPER: Well, we have an expert (Dr. W. C. Rose) in the audience who knows a lot more about this than I do. Certainly, from the work that has been done on adults, it's evident that we can maintain people in nitrogen balance with the protein replaced by purified amino acids, as long as there's adequate extra nitrogen. With regard to the extra caloric requirement, I think everyone who's worked on protein nutrition has discussed it and asked similar questions. I don't know of a satisfactory answer to it.

DR. GYÖRGY: It still indicates, in my opinion, that for efficiency, the protein is still better than its components.

DR. HARPER: Yes. I don't like the term 'efficiency'. I'm not sure that there aren't some physiological factors that enter in here which we don't know anything about. There's a tremendous osmotic effect of a large quantity of amino acids. Can a subject eat enough amino acids during the growing stage to maintain maximum growth? Certainly in some of the animal experiments extremely good rates of growth have been observed. Some of our results differ from those of other laboratories in that, in every case, when we have used amino acid diets, we still have a gap between groups of animals receiving protein, and those receiving only amino acids. The latter grow less rapidly.

DR. GYÖRGY: Well, I'm enough of a teleologist to think protein has been put in food and not amino acids because it is a more efficient way of supporting life.

DR. HARPER: I'm enough of a rationalist to think that animals have survived because they are able to utilize protein to get their amino acids.

DR. WATERLOW: I would like to ask Dr. Harper why he thinks there is an increased need for limiting amino acids under conditions of imbalance. What is the mechanism?

DR. HARPER: It was suggested in a report some time ago that an excess of amino acids that can't be used for protein synthesis must be discarded by the animal, and that this may stimulate the catabolic activity of the animal and thereby cause a loss of a small quantity of the amino acid that is the most limiting. This, I think, is a good working hypothesis.

DR. GYÖRGY: Vegetable proteins are generally low in lysine, and lysine is one of the limiting amino acids. Will enrichment of wheat protein with lysine improve the biological value of wheat protein or are some other factors also important?

DR. HARPER: We know very well from our animal experiments, for example, that the biological value of wheat proteins are improved when they are supplemented with lysine. I don't think there is any question about it, but there are other factors to take into account. We need sometimes to increase the levels of the other amino acids or to raise the protein level. Even then we may have other amino acids limiting; but certainly we can improve the biological value of the protein if we supplement it with lysine.

DR. GYÖRGY: What I had in mind, refers to recent studies of Valyasevi, Kaye and Barness on the effect of fortification of wheat with lysine. It has been shown by these investigations that the addition of lysine alone to wheat had only slight beneficial effect on the utilization of wheat protein. Further addition of potassium will improve not only assimilation but even absorption of wheat protein. Therefore, there are many factors in food which have to be taken into consideration.

DR. SCRIMSHAW: I have another point that I want to bring out. As Dr. Ricardo Bressani has demonstrated at these meetings, there is no doubt that supplementing wheat protein with lysine for children recovering or recovered from kwashiorkor, results in a very marked improvement in nitrogen retention to a level very close to that retained with a similar amount of animal protein. To obtain these results you have to add wheat

gluten and end up with a diet which is very difficult to feed. In other words, the supplementation of wheat flour with lysine will undoubtedly improve the biological value of the protein, but it does not solve the problem of getting enough protein into children, existing on predominantly cereal diets, to prevent protein malnutrition from developing, and to enable them to resist the stress of infections.

DR. GYÖRGY: I might say if vegetable protein is the sole source of protein, amino acid fortification would be very welcome, an improvement on this product, would you agree with that?

DR. SCRIMSHAW: If we are referring to diets in which there is a large amount of wheat consumed, lysine will help; if we are referring to diets with corn, it takes tryptophan as well as lysine. If you are referring to mixed vegetable diets, it has been demonstrated that it is perfectly practical to combine vegetable proteins or vegetable protein concentrates in such proportions as to get a mixture of high biological value.

DR. GYÖRGY: If we state the source of protein, wheat, then would you agree?

DR. SCRIMSHAW: Yes. If you are going to send large quantities of surplus wheat into an area with very poor, predominantly vegetable diets, would you suggest that it be supplemented with lysine?

DR. GYÖRGY: Then, I would have to know whether the amount of wheat was highest in proportion to the other proteins in the diet, and this would influence the decision whether I use it or not; maybe that would not be enough.

DR. SCRIMSHAW: I might add that my major qualification would be the cost, i.e. whether the added cost would be justified by the improved nutritive value.

DR. GYÖRGY: That's a very good point, but this is outside of purely scientific considerations.

We are coming now—to the problem of protein malnutrition and infection. The following questions received from the floor may be lumped together: *a*) If we seek to relate nutrition and infectious disease, why should we focus on protein? *b*) Can protein deficiency affect the prevalence of infectious disease? *c*) Can protein status affect the incidence of infectious disease? *d*) If the coexistence of protein deficiency and infection result in death, shall death be attributed to one or the other, or both?

DR. SCRIMSHAW: The answer to the first question is simply that this symposium was announced to be on protein requirements, and I tried to compile the references and comments that pertain to protein. However, even in the introductory remarks, it was pointed out that infection is directly associated in Indonesia with the development of severe xerophthalmia, keratomalacia and blindness due to vitamin A deficiency. Very striking also are the accounts of persons who were prisoners of war in Japanese prison camps in the Far East, that they managed to get along all right, to avoid edema and other complications until an infection came along; at certain periods of prison camp life, an infection of any sort was almost a seal of death.

Thus, infection is certainly involved in precipitating a great many other deficiencies besides that of protein; conversely, many vitamin deficiencies increase the severity of infections. These facts are largely overlooked here in the United States because they are of secondary significance, but under the critical conditions of prison camp life and persons living in technically underdeveloped areas they may become important.

Secondly, from the evidence we were able to review, there was no indication that malnutrition influenced the initial invasion of the host by a pathogenic organism. Therefore, it did not influence the incidence of disease. It would influence prevalence, however, since prevalence refers to the number of cases found at any given time; therefore, a longer duration of an illness would add to the prevalence. The published information and the experiences in technically underdeveloped areas indicate that malnutrition, under certain circumstances, influences the duration and severity of infection, not the incidence. Increase in the severity and duration prolongs the period of nutritional stress and, as mentioned previously, can result in kwashiorkor. Even with virus infections a secondary bacterial complication may be potentiated by malnutrition and end in death.

As to the last question: "Which comes first, the hen or the egg?" The concept of synergism avoids the dilemma. If malnutrition and infection act together to give an end result which neither alone would produce, it is of academic and not practical importance to determine which is the primary and which is the precipitating factor. I am trying to stress that, from a public health point of view, the end result is usually worse, when malnutrition and infection are combined.

DR. GYÖRGY: I may add that it was Dr. Alan Gregg, who first showed that hookworm infestation is due to malnutrition in Brazil, and with improved nutrition hookworm infestation has been very greatly reduced. I think in very many instances, one can say, as we have always said in pediatrics, that malnutrition is first and lowered resistance second, but I wouldn't like to argue too much about it.

DR. SCRIMSHAW: I was using infection in the broad sense to include infection with parasites of all types, as well as with bacterial, rickettsial and viral pathogens.

DR. GYÖRGY: Inasmuch as we are dealing here

with the assessment of protein requirement I would like to ask Dr. Waterlow, what enzyme measurement would be most informative in assessing protein requirement.

DR. WATERLOW: You mean what single enzyme measurement?

DR. GYÖRGY: Yes, what single one.

DR. WATERLOW: Well, that's a question I asked myself about 5 or 10 years ago. I think it was very naive. I've come to the conclusion it's a question which shouldn't be asked. A single enzyme is an artificial concept, familiar perhaps to the biochemist; but in terms of physiology, enzymes do not work singly, and we shouldn't suppose that the effect of protein loss from tissues would be a specific effect on one particular enzyme.

That's why, in our work, we went on to consider more highly organized systems, working on the empirical idea, that the more organized a system is, the more easily it might be disorganized. We seem to be finding more changes in systems which depend on a whole chain of enzymes, such as oxidative phosphorylation or phosphate incorporation into its phospholipids, than in single enzyme systems. It seems to me that this is a more physiological approach; to ask for a change in one specific enzyme is asking too much.

DR. SCRIMSHAW: I am sure Dr. Waterlow entirely agrees that although there are a variety of biochemical changes associated with kwashiorkor, these do not seem to be particularly useful in detecting the early stages of protein malnutrition. We have all looked for biochemical changes in what might be called the pre-kwashiorkor stage, but by the time alkaline phosphatase or amylase activity or serum proteins are lowered, there are clinical signs such as edema and hair changes which make the early stages of kwashiorkor clinically recognizable.

DR. GYÖRGY: Another question I would like to ask. I should put the question to Dr. Hegsted, but he already has the answer in his contribution; therefore, he will not be able to change it. I hope you, Dr. Scrimshaw, will be able to change it. The statement was made: "Kwashiorkor is seen in areas where the protein content of food is very low. This is probably protein deficiency primarily. In general, however, investigators in the field have failed to identify the role of protein in the etiology of these conditions." Then, somewhere later, the relation of calories and protein was discussed: How much of it is caloric under-nutrition, and to what extent is it protein deficiency?

DR. SCRIMSHAW: We could not be more diametrically opposed in the choice of words on an issue.

DR. GYÖRGY: I knew it.

DR. SCRIMSHAW: I will question first the implications of the correlations between kwashiorkor and calorie deficiency given by Dr. Hegsted in his text. Kwashiorkor can occur with all of the clinical signs and symptoms, and all of the biochemical changes in the presence of an abundance of calories. This is exemplified by the sugar-baby type of kwashiorkor described in Jamaica, and occasionally seen in other parts of the world. Nevertheless, kwashiorkor, as it usually occurs around the world, is associated with a relative deficiency of calories. If you want to give the syndrome a name, you can call it the 'marasmic type' of kwashiorkor. I suppose this type accounts for 99% of kwashiorkor, so that any attempted correlation between caloric deficiency and the presence of kwashiorkor will come out strongly positive as will also a correlation with protein intake.

The opposite extreme from the sugar-baby type of kwashiorkor due to an abundance of calories and relatively low protein, is starvation, where the child receives neither calories nor protein. Partial starvation in a child results in marasmus. The evidence is not all in but that which is available from cases that seem to be due to an equal deficiency of calories and proteins, i.e. partial starvation, is that the biochemical changes and clinical signs of protein deficiency do not occur. You do not have lowered protein; you do not have edema; you do not have fatty liver; you do not have enzyme changes in marasmus.

The picture is confused, however, by the fact that, under natural conditions in underdeveloped areas, a child may go through a period of marasmus and then, perhaps, because of the added stress of infection, become relatively more deficient in protein and develop some edema. If he gets over his infection, he may become a case of marasmus again. A variety of things may keep these children on the border line between marasmus and kwashiorkor, moving back and forth; furthermore, after several weeks of treatment, when the edema is gone, the liver fat has disappeared, the skin lesions are clearing up, the apathy and anorexia are no longer obvious, there is nothing left except the past history of this child to indicate whether he entered the hospital with marasmus or kwashiorkor. Nevertheless, to say that the role of protein in kwashiorkor has not been identified is a statement that most workers would deny vigorously.

Another basis for denying it, although this is also subject to some discussion, is the fact that we can bring about an initial improvement of all of the signs and symptoms of kwashiorkor, including the biochemical changes, through protein alone, or casein hydrolysate, or even, under suitable circumstances, a mixture of 21 amino acids or 9 amino acids made to simulate the com-

plete amino acid pattern or the essential amino acids of milk. Vitamin deficiencies soon become limiting, but initially, at least, these signs and symptoms do respond to protein.

In summary, the relation of calories to the protein deficiency in kwashiorkor, far from being unknown, is well understood, although very complex under natural circumstance. We think that the reason that protein deficiency changes are not seen in marasmus is that the child consumes its own body proteins to match the very low calorie intake.

DR. GYÖRGY: Can anybody else answer the question: what is the difference between hunger edema and kwashiorkor, or is that known? Dr. Widdowson, would you comment on it?

DR. WIDDOWSON: I think in both of them obviously the calories and protein are involved. Edema is a rather non-specific disease. Hunger edema may be induced by calorie deficiency alone, but kwashiorkor could never be produced by calorie deficiency alone.

DR. GYÖRGY: I agree with you. Dr. Watkin, we come to you. You stated in your paper that loss of nitrogen following injury, infection or tumor may be reduced, at least to some extent, through dietary means. Does a normal individual (not in state of malnutrition) who suffers from injury, trauma, burn, or infection, require as an urgent measure, high protein intake from the start, right up until the injury or infection, is over? The second question: what do you do with a person, malnourished, maybe even protein deficient? Would you again start right away with high protein intake or would you be afraid of renal disturbance, excretory defects, or difficulties, and would you, just like in liver diseases, be willing to wait a few days before you gradually increase the protein intake? The third question: whether in animals in which you study wound healing, or in elective surgery in man, does preoperative, preinjury high protein diet improve the course of healing?

DR. WATKIN: Let's go to No. 1 first. I think in the normal individual, who is subjected to—we can use the term loosely—a moderate injury, there's probably not going to be a significant difference in his course whether he is hyperalimented with protein or whether he's given approximately what he would take if he were to select his own diet. However, if he has a truly severe injury, where he would be losing 12–14 gm of nitrogen a day for a prolonged period, then I think that he will do better when given larger amounts of protein than he would probably eat ad libitum, assuming that he has no complication of his injury which contra-indicates protein administration.

Now, this matter of complications leads into the next question which was what to do with a malnourished person. Rarely do you see a malnourished patient who is suffering solely from a failure to ingest an adequate amount of protein. The malnourished person comes to you because, for example, he has been a prisoner of war, during which he has been subject not only to protein depletion but also to caloric undernutrition, to physical and emotional stress of one kind or another, to infections and so on. He may be a patient who comes with cancer or severe liver disease or some chronic infectious ailment. In any event, he does not represent a normal individual or an experimental subject who has been deprived of one nutrient, namely protein. Therefore, I consider the problem of the complications in these malnourished people first.

Obviously, if he is a man who has severe liver disease and I am in danger of pushing him into hepatic coma if I give him a high level of protein, I would want to be very cautious in the administration of protein; if he has chronic renal disease, a high protein intake would increase his blood nonprotein nitrogen and potassium (since most available protein foodstuffs include potassium). In both situations, I would not care to give him a high protein diet until the underlying complication had been corrected if possible.

If he is an individual who has been on a low nutrient regime for a long period of time, say as a result of having had a cancer, or having merely been living under circumstances where he did not get enough to eat, if he has been subject to infection, and so on, an abrupt increase in the amount of calories and protein in his diet may produce rehabilitation edema, and a failure on his part to adjust his enzyme systems, if you will, to the sudden load which has been thrust upon him.

We have seen occasionally this type of thing manifest itself in a very bizarre fashion in cancer patients where a malnourished individual given a high caloric, high protein regime suddenly develops a marked hyperlipemia. Whether this happens in other diseases is something that I'd like very much to know.

To summarize, when dealing with someone who is chronically malnourished, there are many dangers inherent in suddenly forcing upon him a high protein intake.

Now, to go to the third point, whether it is a good idea to, as the surgeons say, build up a patient preoperatively. There is certainly evidence on both sides of the fence here, and to come out with a concrete recommendation at this point, would probably be an unwise thing for me to do at this time. As an internist, I will say this much, however: if a patient is going to need elective surgery at some time, let us say 3–6 weeks in the future, and he is one of these mal-

nourished people that we were just discussing, then it is the responsibility of the surgeon to see to it that he is slowly brought up to a state of at least moderately good nutrition before attempting an elective procedure; if the individual comes to you for an elective procedure in a good state of nutrition, without any serious complications, (which may have been the thing, of course, which led to his coming to you in many instances), then I see no reason for giving him an unusually high level of dietary protein.

The practice of administering high protein feedings may induce complications in the postoperative phase as well as in the preoperative phase, and it's something that has to be done with a high degree of caution. Very often these high protein regimes also include high concentrations of electrolytes; they are administered by tubes to unconscious patients who cannot object to or reject them. Hypernatremia, hyperchloremia, and dehydration may be serious complications unless due regard is paid to the water balance and water requirements of the patient.

DR. GYÖRGY: I would like to ask you one final question. Do I understand you, first, that even in traumatized patients, you would not start right after the trauma with very high amounts of protein, but maybe you'd do it slowly because of the possibility of complications which you mentioned, and the well known one, edema, and I might mention renal insufficiency which might occur after trauma; and second, is there any place for very high protein at any time, can you bring up the protein reserves by giving 300-400 gm of protein per day? Is there any reason to give more than 150 gm of protein at any time unless you have diarrhea or some other extraneous losses, for instance in acute burns?

DR. WATKIN: Well, there are certain situations where you can certainly give 150 gm of protein.

DR. GYÖRGY: I said 300–400.

DR. WATKIN: I think one has to bring into the discussion here the problem of calories. If you will look at the chart taken from Dr. DuBois' work on page 1162, you'll see an indication of the caloric requirement on a fixed protein intake of an individual with typhoid fever. This is a good example of exactly what we're discussing here. The caloric requirement enabling a patient with typhoid to maintain a nitrogen equilibrium is sometimes two times or more the actual caloric expenditure as measured by indirect calorimetry.

Therefore, I think, in many instances, it is more important to give more calories than it is to give an excessive amount of protein assuming the protein intake is already high. One of the best studies pointing out the value of protein, I think, is one that was done at the Walter Reed Medical Center by Dr. Plough and others in which they related the level of dietary calories to the level of dietary nitrogen in patients with chronic liver disease. They were able to demonstrate very nicely that if they gave a low protein intake (and this was not very low either—this was in the order of 70 gm a day or so) and a high calorie intake, you got a marked increase in the amount of fat in an individual and gain in weight but you didn't get the retention of much protoplasm. However, if you increased the protein to a level of 150 gm or so, keeping the calories constant, you did get laying down of protoplasm.

We've had experience in 2 cancer patients (and incidentally these 2 we regarded as almost 'normal' controls because their cancers were inactive) who were given truly high protein diets (something in the order of 200–250 gm a day to a 70 kg man). In these individuals the results were universally bad. They developed diarrhea; they showed signs of nitrogen retention as judged by blood nonprotein nitrogen and blood urea nitrogen concentrations, and, when renal functions were measured, decrease in tubular function and glomerular function. So, on the basis of the evidence that I'm aware of, there are very, very few situations where hyperalimentation with protein, i.e. above 150 gm a day in a 70 kg man is truly justified. Mention should be made, though, of the possible 'intolerance' to carbohydrates of the acutely injured patient. The optimal dietary protein: fat:carbohydrate ratios for the severely injured are not yet known.

DR. WATERLOW: Dr. Watkin, I would like to ask you a question. If a person comes with low protein reserves, but without a complication such as neoplasm, according to what you say, he has a smaller metabolic loss. Doesn't this influence his recovery? I notice in your first chart, one patient had a skin burn of very severe degree, he received no nitrogen, yet he seemed to recover just as well as the one who did receive nitrogen. On the other hand, Dr. Scrimshaw states that having low protein reserves does affect the clinical course. If you don't answer the question I think you should do clinical surgical research in an under-developed area.

DR. WATKIN: What do you mean?

DR. SCRIMSHAW: This one of the things that always complicates discussions of this sort. Dr. Waterlow and I are talking about a degree of protein depletion hard to conceive of here in the United States, even in terminal cancer patients; that may be part of the difference.

DR. WATKIN: Well, we're dealing, in the patients in the charts, with two members of the U. S. Army, and I think by and large they have experienced, during their military careers at least, excellent nutrition. I think that all I can say in

answer to your question is that we'd all love to come to Jamaica or Guatemala if you could arrange it.

DR. GYÖRGY: May I now ask Dr. Ross a few questions: what are the advantages of maximal growth rate? This will lead you to the problem of calories, and to our special problem, protein.

DR. ROSS: If this question is examined from the point of view of life span and age-associated diseases several impressive correlations are noted. In our studies, animals which had the highest growth rate had the lowest life expectancy and the highest incidence of age-associated diseases. Spontaneous tumors, kidney disease and vascular disturbances were common pathological findings. When growth rates were retarded there was enhancement of life expectancy and, which is even more remarkable, a reduction in incidence of several degenerative conditions. As stated in my formal presentation, the influence of the caloric level on these diseases is modified by the protein intake. Comparison between early growth rates and late occurring diseases is not limited to populations differing as to their nutritional regimens. An analysis of growth rates within one group of rats on the same diet strongly suggests that those rats with the highest rate of growth have a much better chance of developing later in life a major degenerative disease than do those whose growth rate is not as great.

Each nutritional set-up appears to evoke different responses at different times. A high protein intake favors a low mortality rate early in life, but a low protein in conjunction with a low carbohydrate intake favors a low mortality rate later in life.

DR. GYÖRGY: I have a question here from the floor. What expectation do you have if you would have changed the food intake or caloric intake, protein intake in earlier life, not after weaning? You have no data?

DR. ROSS: I would save the next 5 years of my work if I had it.

DR. HARPER: Well, is there a suggestion from what you have done that a high food intake and a high growth rate during the early growing period is best because fewer animals succumb to disease under these conditions.

DR. ROSS: On the surface this would appear to be desirable, but there are several indications that the early nutritional state, with its attendant metabolic patterns, may exert some influence on the events that are manifested much later in life.

DR. GYÖRGY: Has it ever struck you that maybe the protein level in your 'low protein' diet was too low and maybe the protein level in your 'high protein' diet was too high in hyperalimentation?

DR. ROSS: Yes.

DR. GYÖRGY: That you mentioned in your introductory remarks.

DR. ROSS: In an examination of the amount of protein allotted, not on the basis of the rat, but on the basis of body size, it becomes apparent that the 'well-fed' rat outruns the protein intake as to further increase in weight; but the ratio of protein intake to body size of the growth-retarded rat remains constant throughout the entire course of life.

It would seem that a higher protein intake, whether it be on the basis of the entire organism or entire body weight, would be beneficial in reducing mortality rate early in life, and that a lower protein intake later would be beneficial during the later phases of life. But the question must be asked again, are we setting up a situation which may determine or influence events in later life?

DR. SCRIMSHAW: This is the one that you still need to answer.

DR. GYÖRGY: Do you think if you would change from a high protein, high carbohydrate diet, to low protein, low carbohydrate diet later, you would get the same beneficial effect that you got with low protein, low carbohydrate from the beginning?

DR. ROSS: That's the same question but there are other data which we cite to indicate that an early nutritional state can influence the reaction of an organism to a subsequent change in diet. To complicate matters, the age factor is also most important. With a change in diet there is first a drop in weight regardless of whether we're going to a higher protein, higher carbohydrate level or to a lower protein, lower carbohydrate level, or a combination thereof. With a change to a high protein, high carbohydrate intake, we find that there is an adaptation mechanism in that these animals show signs of recovery in weight about 5 or 6 weeks later and the growth rate then parallels that of the animals fed on the same diet from weaning age. On the other hand we have situations where a change in diet provokes an entirely different pattern of adaptation in these cases. There is also a weight loss following a change in diet and after a given period of time the animals are able to maintain themselves at a lesser weight but are not capable of further growth.

If the same diets are used at an earlier age, i.e. during weaning, growth does occur and proceeds beyond the weights of the rats which had a different earlier nutritional history. It would appear then that age is just as important a factor in this story as the diet. No data are yet available to indicate whether multiple shifts in diet can be successfully made and whether beneficial effects will result.

DR. GYÖRGY: Now, I think we've reached a stage where I'll ask Dr. Waterlow to summarize

what he heard and then we'll see what we've accomplished.

DR. WATERLOW: I think all who remain in the audience will agree that my task is almost impossible, and I'll try not to emphasize my own point of view. I shall confine myself to the points which were most controversial.

The first point considered was the question of the constancy of tissue composition, or of body composition in terms of the nitrogen content of lean body mass. I think the panel in general agreed with Dr. Wallace that this was true, in the sense that the ratio of nitrogen to water is constant under many conditions of diet, and at most stages of life. In the newborn and the premature there is a higher tissue water content and a lower nitrogen:water ratio, which is made up by the process of maturation during the first year of life.

There was no information on whether this chemical maturation process could be speeded up.

The panel on the whole seemed to agree that in protein depletion there is no change in this constancy of the nitrogen content of the lean body mass. On the other hand, some felt that this doesn't cover all the factors and that there may be a change in the pattern of the different proteins. It is not enough to take only two constituents, water and nitrogen; if you use some other constituent as a reference base, such as desoxyribonucleic acid, you find that there is a change in the protein:DNA ratio. This doesn't controvert what Dr. Wallace said, but is simply an amplification, and a further point.

This brings us to the question of the protein reserve or labile protein. Again, I think that the panel agreed that there is such a thing, in the sense that if an animal or a human being is changed from a high to a low protein diet, nitrogen is lost and the organism then comes into equilibrium at a lower level.

But the question arises: what is this loss of nitrogen? What does it represent? Where does it come from? Various possibilities were considered; that there was an actual loss of cells, a shrinkage in the size of cells, or a fall in the concentration of nitrogen or protein in the cells.

The third possibility has been considered, and on the whole rejected. So, we are left with two: a loss of cells or a shrinkage of cells. The method of expressing the protein content in relation to DNA tends to indicate that there is a loss of protein from cells which become smaller in size but do not disappear.

The panel agreed that this is a dynamic concept and that there is no fixed, separate reserve protein; it's simply that some proteins are more labile than others. This again brings us back to the point that I made before, that when the protein reserves are depleted, you probably get an alteration in pattern of the cellular proteins.

The next controversial problem was that of protein requirements. I think the panel were agreed that this has to be approached in three steps: the first is the basic requirement of protein, in terms of any reference protein that we may choose. Here Dr. Hegsted introduced the concept that this basic requirement can be calculated from the minimal nitrogen excretion in relation to basal metabolic rate. This to me was a new idea and I don't think the panel discussed it as fully as they should. The point was made, but perhaps not brought out clearly enough, that on the whole, when you assess the basic requirements in this way, the results agree well with the evidence obtained from growth studies in children and maintenance studies in adults. Where they don't agree, Dr. Hegsted tried to explain why they shouldn't.

The second stage in estimating requirements is to extrapolate, as it were, from the basic protein to other proteins with different amino acid compositions and nutritive values. The third stage is to try to make some allowance for stresses of various kinds.

The second stage brought us on to discuss the FAO reference pattern. Is this a good and useful idea? I think again everybody agreed that we must have some system for converting one protein into terms of another. Whether you use an artificial reference pattern (artificial in the sense that it doesn't represent anything actually eaten) or whether you use some natural protein, was a matter of controversy. The controversy does not go very deep because everybody is agreed that you have to have some standard of reference, and it doesn't really matter very much what it is, as long as you make it clear. The FAO reference pattern is useful in that it directs attention to amino acid needs, and it raises the problem of whether protein needs are to be thought of entirely in terms of amino acid needs.

Then, we went on to consider what happens with imbalance in proteins. I think it is accepted by all that you have to feed more of an unbalanced protein according to the amount of limiting amino acid it contains.

Dr. Harper made a very important point here, that even this calculation based on the limiting amino acid may not give you the right answer because excess of an unbalanced protein increases the need for that limiting amino acid. Dr. Scrimshaw brought up practical examples of the same kind of thing happening, where calculation based on the 'ideal protein' pattern didn't seem to apply in practice. I think again everyone on the panel will agree that this is a key point for further research in an important practical field.

I cannot summarize in any detail the discussions on malnutrition and infection, or malnutrition and resistance to stress and surgical operations—Dr. Scrimshaw's and Dr. Watkin's contributions—nor the final one of Dr. Ross on the effect of different levels of protein and calories on growth rates, development of congenital and idiopathic diseases, and mortality, because, on the whole, these were less controversial. The great value of these contributions was to show us, by the absence of controversy and the large number of questions, how very little we know about these subjects. As Dr. Scrimshaw pointed out, we recognize the very severe effects or, the combination or synergism, as he called it, of malnutrition and infection; but I don't think we know how this comes about, and I don't think that we are able to relate it to the derangement of metabolism and the nitrogen losses described by Dr. Watkin.

Dr. Ross' contribution was so unique that it didn't arouse very much discussion, but only a certain amount of horror from those of us who thought maybe we'd been eating too much.

DR. GYÖRGY: Well, thank you very much, Dr. Waterlow. We would like to ask the members of the panel whether there is any very vehement disagreement that they would like to put on record, or whether the members—just as I am—are grateful to Dr. Waterlow for this summary.

If I don't have any disagreement from the panel, or from the floor, the audience, I will close the symposium.

(Whereupon at 4:20 P.M. this session closed.)

AMERICAN PHYSIOLOGICAL SOCIETY

Symposium on Biological Rhythms[1]

Chairman: H. S. MAYERSON

PHOTOBIOLOGY AND BIOCHEMISTRY OF CIRCADIAN RHYTHMS IN NON-PHOTOSYNTHESIZING CELLS[2]

CHARLES F. EHRET

Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois

WHILE IT IS TRUE that daily periodic phenomena in living systems were recognized even by the ancients, it is only within the past 10 or 15 years that observations have been extensive enough and conditions precise enough to justify the conclusion that daily rhythms continue to persist in many organisms even in the absence of the usual ambient variables of the laboratory (1–3). Thus the general demonstration of daily rhythms in organisms is a discovery of contemporary biology. The phenomenon has been observed in most of the major phyla of the plant and animal kingdoms, with the conspicuous exception of the bacteria.

An important point regarding the frequency of such rhythms is that while a single cycle approximates a day's length, it is usually observed to be significantly different from 24 hours. The descriptive term 'circadian' (*circa dies*) (4, 5) acknowledges this fact that constitutes one of the strongest pieces of evidence favoring an endogenous (intra-organismal) timekeeping mechanism. Brown (3, 6) on the other hand has preferred the view that a 'cosmic' stimulus external to the organism accounts for its daily rhythm.

One of the most thoroughly studied organisms, the unicellular protozoan *Paramecium bursaria* is also the smallest and simplest non-photosynthesizing form in which, to date, such rhythms have been clearly shown. The phenomenon observed at the cellular level in *Paramecium* appears in many respects to be essentially the same as that seen in more complex systems. It is therefore reasonable to hypothesize that basically similar timekeeping mechanisms are involved, that these are subcellular in location and organization, and that they are as fundamental to a type cell as its nucleus. The principal goal of our studies has been to find and characterize such a mechanism.

This species of *Paramecium* is normally green in nature owing to the presence of hundreds of algae of the genus *Chlorella* in its cytoplasm. In 1939, Jennings (7) had observed that in varieties (or syngens) 1 and 2, mating normally occurs in the daytime but not at night. We then showed that the time of mating is readily influenced by light, that the algae are not essential to the photosensitive reaction and that the rhythmical occurrence of the mating reaction persists for days in total darkness in the absence of such other variables as temperature and nutrition (8, 9). In most of the physiological studies, the experimentally-manipulated cells were chlorella-less, while the testers of complementary mating-type were chlorella-containing. Thus within the masses of agglutinating cells that result from mixing reactive experimental cells with reactive tester cells, it is always possible to recognize the two types. The recorded datum is the number of chlorella-less cells agglutinating at ten minutes after mixing with testers.

TEMPERATURE EFFECTS

The influence of temperature upon some properties of the rhythm (10) measured by the

---

[1] Atlantic City, New Jersey, April 16, 1959.

[2] This work was performed under the auspices of the U. S. Atomic Energy Commission.

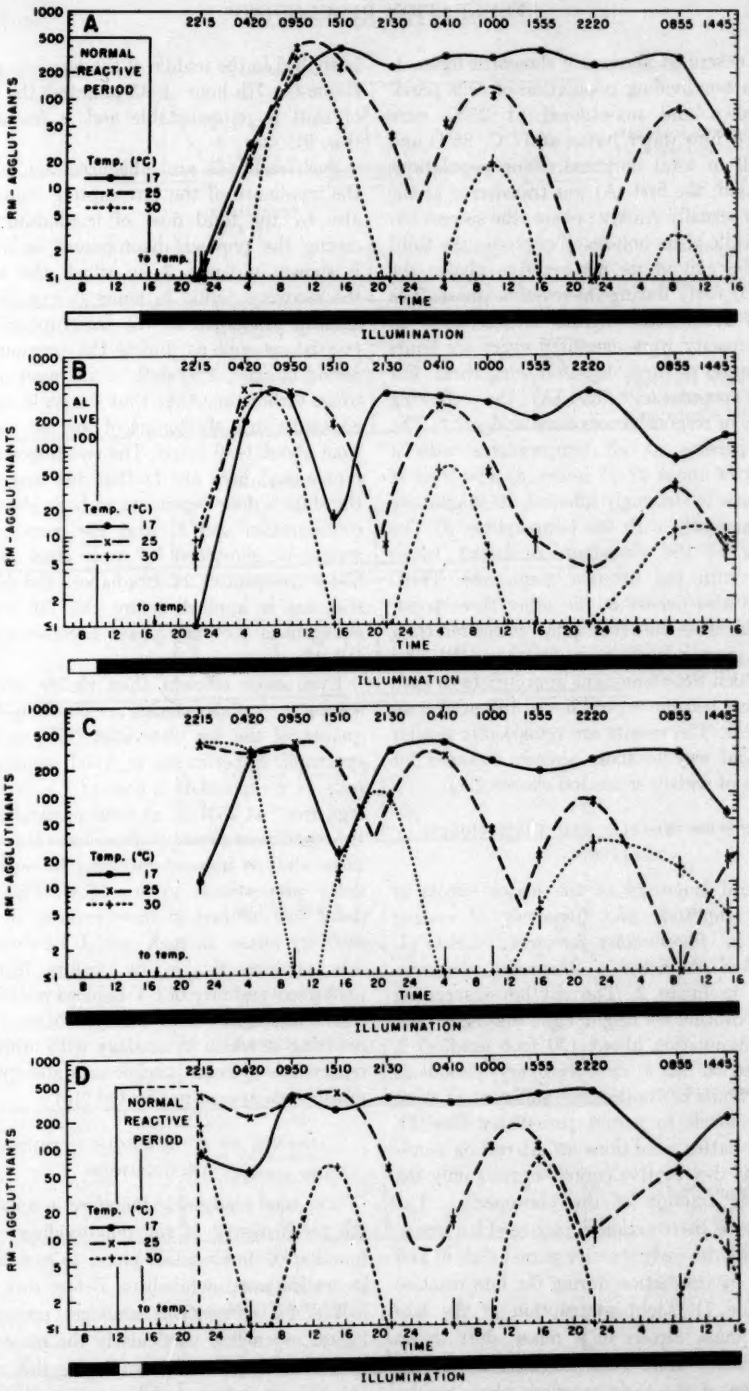


FIG. 1. Influence of temperature upon some properties of the circadian rhythm of mating capacity in *Paramecium bursaria* (chlorella-less).

methods described above are shown in figure 1. Cells of a nondividing population of cells previously grown and maintained at 25°C were transferred into water baths at 17°C, 25°C and 29.5°C all in total darkness. Four populations were treated; the first (A) was transferred at the end of its sexually reactive phase; the second (B) in the middle of its non-reactive phase; the third (C) at the end of its non-reactive phase; the fourth (D) early during its reactive phase. The effects of these heterothermal transferences on mating capacity were measured every six hours for the period of three days following them. For the first population (fig. 1A) the following properties of responsiveness were evident: *1)* The rhythm persists at all temperatures with a frequency of about 22–23 hours. *2)* The level of the response is strikingly affected, its magnitude varying inversely with the temperature. *3)* The resolution of the circadian oscillation varies inversely with the reaction magnitude. These properties also persist in the other three populations. In these however some phase-resetting occurred, particularly in populations (B) and (C), in which the oscillations appropriate to each temperature became several hours displaced from one another. The results are remarkably similar in a general way to those already reported for organisms of widely separated classes (11).

ACTIONS OF VISIBLE AND ULTRAVIOLET RADIATIONS

A general summary of the major effects of light on amplitude and frequency of mating capacity in *Paramecium bursaria* (variety I, chlorella-less animals) is illustrated diagrammatically in figure 2. The rhythm-suppressing action of continuous bright light (daylight fluorescent illumination about 150 foot candles) is represented on line 4; dark 'recovery' periods as brief as 2 hours in duration are sufficient at these irradiance levels to permit periodicity (line 5). Under the latter conditions an interesting corollary is that the reactive period occupies only the small early fraction of the photoperiod. The magnitude of the reaction is increased by irradiation during the early reactive period (line 6) and decreased by irradiation during the late reactive period (line 7). Light-interruption of the late recovery phase results in a phase shift to an earlier relative time (line 8) whereas similar interruption of the early recovery phase results in a shift to a later time (line 10). If the stimulus is applied in the middle of the recovery phase (at about the 7th hour ± 45 minutes) the direction of shift is unpredictable and is frequently nil (line 9).

Such reactions are influenced not merely by the irradiance of the interrupting stimulus, but also by the total dose of irradiation received during the previous photoperiod as well. This is shown in figure 3, in which the length of the recovery period in hours (y-axis) is plotted against irradiance in the interruption impulse (x-axis) as well as during the previous photoperiod (z-axis). The shift in the onset of mating to an earlier time (like that shown in fig. 2, line 8) results in a shortening of the recovery phase from about 14–9 hours. The two important facts represented here are *1)* that the magnitude of the shift is dose dependent in both phases under consideration and *2)* that the recovery period cannot be shortened by more than about 4½ hours irrespective of irradiance: indeed, if the stimulus is applied before the 7th hour, the endogenous recovery phase becomes prolonged (12, 13).

Even more efficient than visible light in resetting the cellular clocks, are the more energetic quanta of the far ultraviolet (UV) end of the spectrum. Experiments in which populations of cells were exposed to a dose of UV (about 6000 ergs mm^{-2} at 2537 A) at some particular time of the reactive or recovery phase have shown that a phase-shift is induced resulting in each case in delay proportional to dose (14). The greatest delay was induced in those exposed during the recovery phase. In each case, UV-induced delay was photoreactivable by visible light. This photorecoverability of UV-induced phase-shifting represents a new and highly efficient form of resetting in which by analogy with other photorecoverable systems, nucleic acid absorption and metabolism appear implicated (15).

ABSENCE OF CONSPICUOUS CYTOLOGICAL OSCILLATIONS

It seemed reasonable therefore in approaching the biochemistry of the non-dividing cell as a function of its circadian phase to look first into its nucleic acid metabolism. Before this, we had looked for correlations amongst grosser cytological organelles, particularly the macronuclear nucleoli. These are known to be rich in RNA (16, 17), are extremely UV sensitive (18, 19), and are normally heterogeneous in form within a

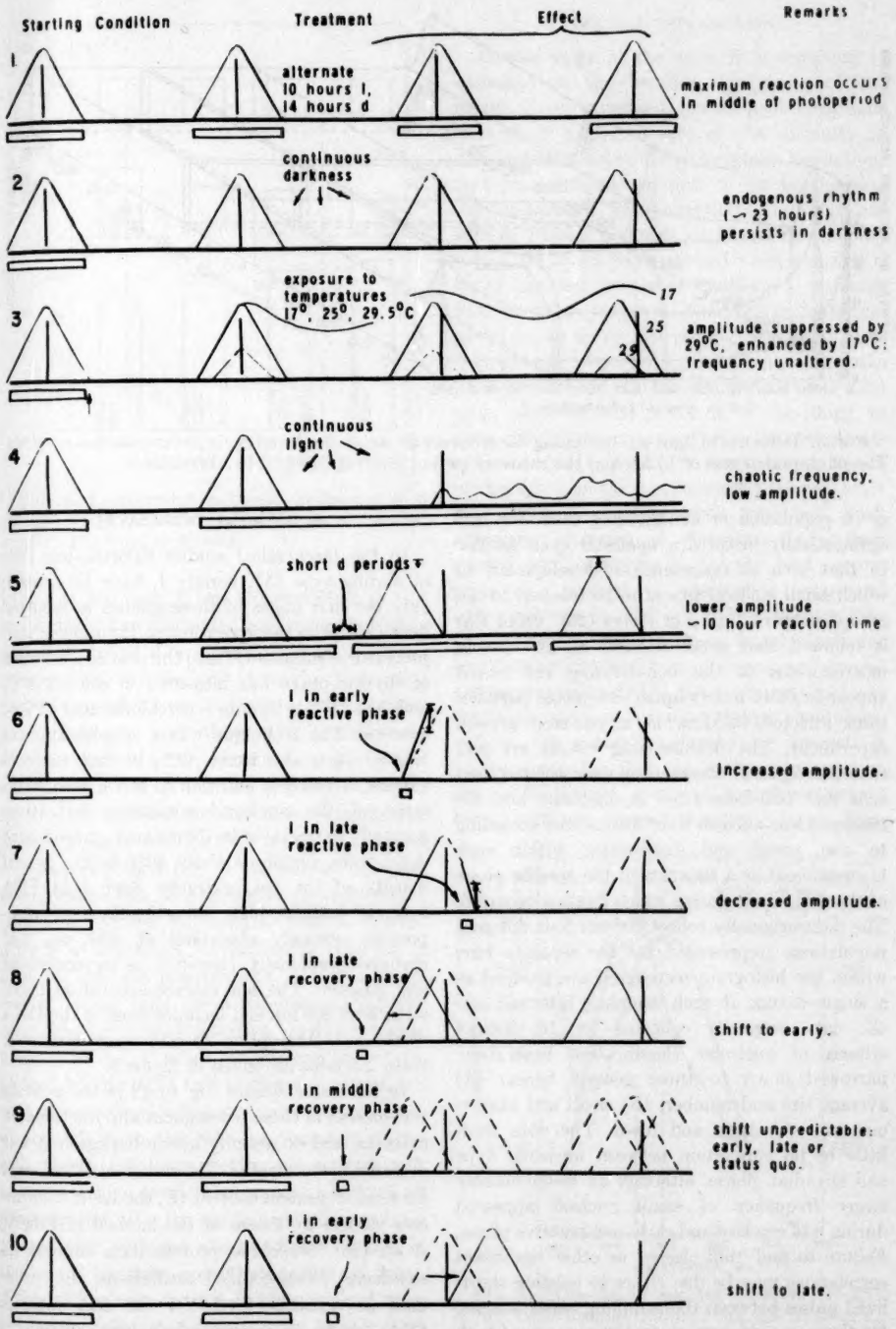


FIG. 2. General summary of the major effects of light on magnitude and frequency of mating capacity in *Paramecium bursaria*.

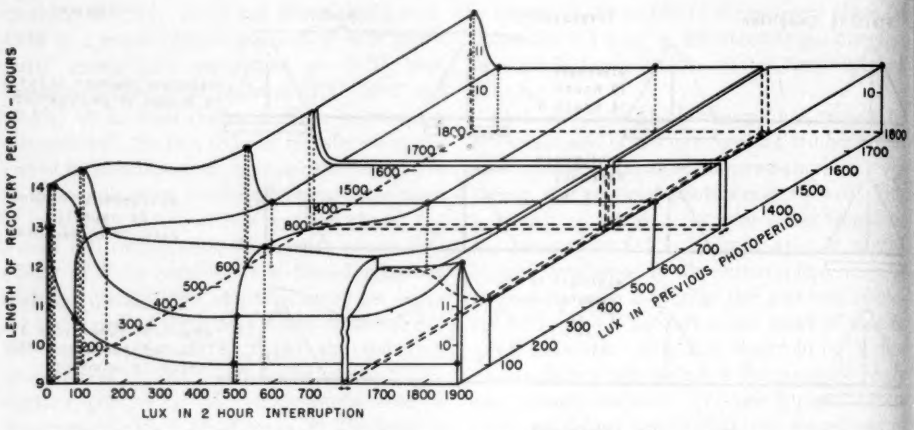


FIG. 3. Influence of light on shortening the recovery phase of the sexual cycle in *Paramecium bursaria*. The photoperiod was of 10 hr. and the recovery period interruption of 2 hr. duration.

given population of non-dividing cells. We had optimistically pictured a nucleolar cycle similar to that seen in macronuclear development in which small nucleoli appear to be released to the cytoplasm periodically in waves (20). From this it followed that small nucleoli in the mature macronucleus of the non-dividing cell might appear in daily bursts much like phage particles from infected bacteria in a one-step growth experiment. The disillusioning results are presented in figure 4. Macronuclei were isolated from cells that had been fixed in digitonin, and the macronuclear nucleoli were then scored according to size, count and distribution within each macronucleus as a function of the specific phase of the daily cycle during which fixation occurred. The data originally collected from four different populations (represented by the separate bars within the histogram rectangles) are grouped as a single datum at each sampling interval; and the data originally collected for 16 distinct criteria of nucleolar classification have been narrowed down to three general types: (A) average size and number, (B) small and numerous, and (C) large and fused. The data show little or no correlation between nucleolar type and circadian phase, although an insignificantly larger frequency of small nucleoli appeared during late reactive and early non-reactive phase. Failure to find 'null classes' or other significant correlations may be due either to existing short-lived pulses between the sampling times selected for these experiments, or to the absence of such pulses.

BIOCHEMICAL CORRELATES

In the biochemical studies chlorella-less cells of mating type (A), variety I, have been used; only the first phase of these studies is reported here (21). The incorporation of the nucleic acid precursor adenine-8-C^{14} into the cells as a function of rhythm-phase was measured in cold (2–4°C) and hot (70°C, 20 min.) perchloric acid (PCA) extracts. The technique was a simplification of that of Ogur and Rosen (22), in that the cold extract included in addition to RNA derivatives some of the nucleotide-coenzyme derivatives normally extractable in dilute acid; indeed such derivatives contribute about 30% to the optical density of the cold extracts. Such cold PCA extracts contain high concentrations of components strongly absorbing at 250 mμ and chromatographically identified as hypoxanthine and guanine. The hot extract absorption maximum is at 260 mμ and includes most of the DNA of the cell. Absorption spectra characteristic of these fractions are given in figure 5.

In the tracer studies (fig. 6) all of the activity was present in these two sequentially fractionated extracts, and no activity above background was detected in the residue. The residue was analyzed for relative protein content (*P*, the Klett colorimeter values) by means of the method of Lowry, *et al.* (23). Since the protein data showed no significant phase-coupled oscillations, they were used as reference levels for the cold extracts (*NAc*, circles, the absorbancy values at 250 mμ) and hot extracts (*NAh*, triangles, the absorbancy

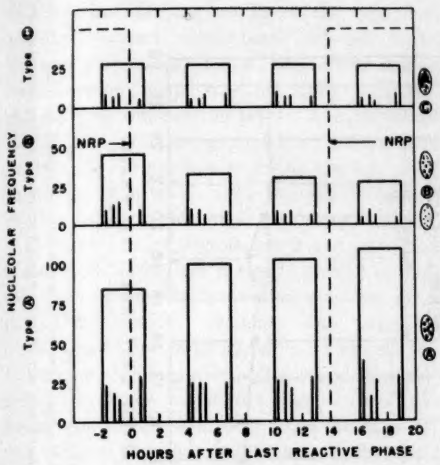


FIG. 4. Relationship between frequency of a specific nucleolar type and phase of the sexual cycle in *Paramecium bursaria*.

values at 260 mμ). When the respective NA:P ratios were then calculated and compared from data collected at 2 or 3 hour intervals in experiments of 24 and 48 hour duration, systematic and characteristic oscillations of these ratios were observed, and these were consistently coupled to some phase identified by the physiological criterion of mating capacity. Similar rhythmical oscillations were also seen in the incorporation of adenine-8-C^{14} into both extracts. An M-shaped curve (fig. 6) characterized adenine incorporation into NAc during the recovery phase, with the greatest increase in relative specific activity at this time. A conspicuous influence of light on adenine incorporation is noted (open circles) when cells are irradiated during the physiologically photosensitive early recovery phase. The NA:P ratios for this experiment are plotted on the right half of figure 6. Correlated with the increased adenine uptake is an increase of about 25% in the NAc concentration of the cells about 4–8 hours before the control cells reach that high level. The gradual rise in NAh concentration after the eighth hour also correlates interestingly with the rise in adenine uptake in the same fraction over that period of time. Although the cells are non-dividing (indeed, they are approaching starvation), some nucleic acid synthesis appears to proceed during this phase.

GENERAL IMPLICATIONS

At this stage of the work it is tempting to speculate on the broader significance of our results. Every investigator in the field of rhythms research is acutely aware of the difficulty in distinguishing between small-systems oscillations that represent the running of the timekeeping mechanism itself, from those that merely correlate with it. Indeed, a simple set of criteria whereby such a clock can be recognized when and if it is found has not yet been established, although some boundary features are implicit in the formal model drawn up by Pittendrigh, Bruce and Kaus (24). On the subject of actual mechanical clocks (in a treatise on another subject) Porter (25) aptly observes that while it is one thing to characterize certain regularities in the movements of the hands of a watch, "it is another matter to predict (without taking the watch to pieces) what the precise mechanism inside may be." Such necessary processes of dissection have been carried out biochemically for the past several years in a number of mammalian organ and tissue systems by Halberg and co-workers (26–28). These investigators have found significant circadian oscillations in many organs and several systems including DNA, RNA, phospholipid and corticosterone metabolism. Although such observations are naturally of intrinsic interest, it appears probable that the systems in question are not the primary source of 'organism standard time,' but are instead receiving signals from

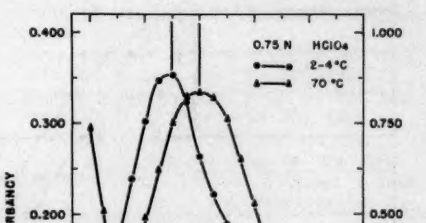


FIG. 5. Absorption spectra of cold (2°–4°C, 20 hr., right ordinate) and of hot (70°C, 20 min., left ordinate) extracts of *Paramecia* in 0.75 N Perchloric acid.

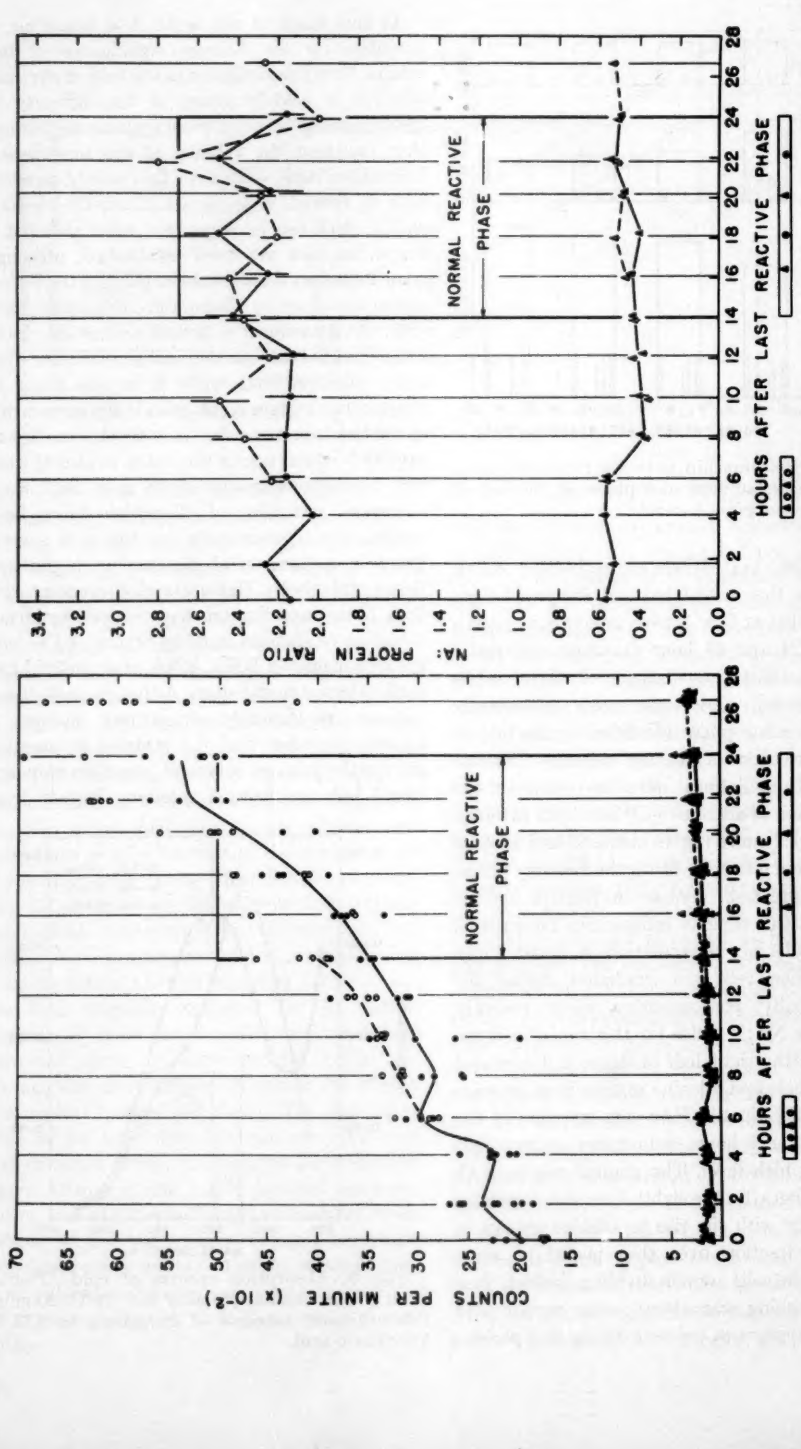

COUNTS PER MINUTE (x 10⁻²)
NA: PROTEIN RATIO
NORMAL REACTIVE PHASE
HOURS AFTER LAST REACTIVE PHASE
NORMAL REACTIVE PHASE
HOURS AFTER LAST REACTIVE PHASE

FIG. 6. Recovery of the label of adenine-8-C^{14} into cold (circles) and hot (triangles) perchloric acid extracts as a function of time and a 3 hr. light interruption (open symbols, and open rectangle below abscissae). The populations of non-dividing chlorella-less cells were first exposed to the tracer for 24 hr. before the origin on this graph. On the right, the ratios of NA:P for cold and hot PCA extracts are given; each vertical bar represents two standard errors of the mean.

another time keeping center in the organism, possibly located somewhere between optic chiasma and hypothalamus (29). Such studies have therefore, contributed few clues to the biochemistry of timekeeping per se. Also, sufficient studies have not yet been made of isolated cells or tissues *in vitro* culture to decide whether every subsystem of a whole organism with circadian rhythm retains within itself such a mechanism.

In the unicellular phytoflagellate and protozoan systems, the basic clock is clearly present in each cell (barring an environmental stimulus of a particular circadian frequency and unknown cosmic origin) and must therefore be subcellular. In the case of *Paramecium* an hypothesis that the clockworks reside within the framework of the nucleic acid metabolism of the non-dividing cell was inspired by the observation of the high efficiency of far UV quanta in resetting phases, and the photoreactiveability of this effect by longer wavelengths. While our experimental findings regarding circadian oscillations of nucleic acid or nucleotide-coenzyme derivatives and precursors are consistent with this hypothesis, they too may be of merely correlative rather than causal significance. However, the very early responsiveness to light, during a dark period, of the incorporation of the nucleic acid precursor adenine, correlates closely to the physiological responsiveness of the whole cell, suggesting a close proximity of this system to something like the basic 'A oscillator' in the coupled oscillator model of Pittendrigh, Bruce and Kaus (24). It is therefore heuristically sound to conceive of these correlations as representing evidence of the biological clock escapement mechanism that we have been seeking—a particularly intriguing idea in the light of the role of purines and pyrimidines in feedback systems of the sort recently described by Gots (30) and by Wyngaarden *et al.* (31, 32). Such relationships further suggest the experimental application of purine and pyrimidine analogs to suppress or stop the normal clockworks. Amongst these of course, would be included the barbiturates, well-known already for effects on wakefulness, and the xanthines (caffeine, theobromine, theophylline) equally well-known for their effects on sleepfulness—as well as on microbial nucleic acid metabolism (33).

It is superficially evident therefore, that the pharmacological control of cellular circadian clocks is theoretically feasible. If this proves to be correct, then the practical applications in organismal control methods will be of great importance. It is immediately proposed that such drugs as those that have already been shown empirically to be effective upon the whole organism, be applied locally to the organism's suspected timekeeping centers, not only so as to increase efficiency of drug action, but especially to reduce the well-known toxicological side effects of such agents. Whether or not complete control of an organism is possible to the point of free-running randomization of all systems so that the organism as a whole is 'clockless' can at present be only a matter of conjecture. Indeed it is equally feasible that the entire economy of organismal syntheses is so integrally bound to a long-time-interval clock that a completely desynchronized organism would be a contradiction in terms and impossible of achievement. Time will tell.

I am grateful to Mrs. Celestia Crawford, Janet Railsback Fraembs, Charlene Loehmann Harrison and Rosemarie Mather Hansen for their technical assistance over many years. I also wish to thank Drs. F. Schlenk, S. Shapiro and E. L. Powers of Argonne National Laboratory for their generosity of time and advice.

REFERENCES

1. Bünning, E. *Die Physiologische Uhr.* Berlin: Springer, 1958.
2. Pittendrigh, C. S. and V. G. Bruce. In: *Photoperiodism and Related Phenomena in Animals and Plants*, edited by R. B. and A. R. Withrow. Washington: Am. A. Adv. Sc., 1959.
3. Webb, H. M. and F. A. Brown, Jr. *Physiol. Rev.* 39: 127, 1959.
4. Pittendrigh, C. S. *Fed. Proc.* 18: 643, 1957.
5. Halberg, F. *Fed. Proc.* 18: 643, 1959.
6. Brown, F. A., Jr. *Fed. Proc.* 18: 643, 1959.
7. Jennings, H. S. *Genetics* 24: 202, 1939.
8. Ehret, C. F. *Anat. Rec.* 111: 112, 1951.
9. Ehret, C. F. *Physiol. Zool.* 26: 274, 1953.
10. Ehret, C. F. *J. Protozool.* 6: Suppl. 1, 1959.
11. Bruce, V. G. and C. S. Pittendrigh. *Am. Naturalist* 91: 179, 1957.
12. Ehret, C. F. Argonne National Laboratory Report No. 5426, 153, 1955.
13. Ehret, C. F. *Anat. Rec.* 122: 456, 1955.
14. Ehret, C. F. In: *Photoperiodism and Related Phenomena in Animals and Plants*, edited by R. B. and A. R. Withrow. Washington: Am. A. Adv. Sc., 1959.
15. Jagger, J. *Bact. Rev.* 22: 99, 1958.
16. Egelhaaf, A. *Arch. Protistenk.* 100: 447, 1955.
17. Schwartz, V. *Biol. Zbl.* 75: 1, 1956.
18. Kimball, R. F. and N. Gaither. *J. Cell. & Comp. Physiol.* 37: 211, 1951.
19. Ehret, C. F. *Radiat. Res.* 3: 222, 1955.
20. Ehret, C. F. and E. L. Powers. *Exp. Cell. Res.* 9: 241, 1955.

21. Ehret, C. F. and R. Mather. *J. Protozoöl.* 6, Suppl. 1, 1959.
22. Ogur, M. and G. Rosen. *Arch. Biochem.* 25: 262, 1950.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. *J. Biol. Chem.* 193: 265, 1951.
24. Pittendrigh, C. S., V. G. Bruce and P. Kaus. *Proc. Nat. Acad. Sci.* 44: 965, 1958.
25. Porter, A. W. *Encyclopedia Brittanica* 22: 109, 1951.
26. Halberg, F., H. Verdmund, E. Halberg and C. P. Barnum. *Endocrinology* 59: 364, 1956.
27. Barnum, C. P., C. D. Jardetsky and F. Halberg. *Am. J. Physiol.* 195: 301, 1958.
28. Halberg, F., R. Peterson and R. Silber. *Endocrinology* 64: 222, 1959.
29. Bullough, W. S. In: *Photoperiodism and Related Phenomena in Animals and Plants*, edited by R. B. and A. R. Withrow. Washington: *Am. A. Adv. Sc.*, 1959.
30. Gots, J. S. *J. Biol. Chem.* 228: 57, 1957.
31. Wyngaarden, J. B. and D. Ashton. *Nature, London* 183: 747, 1959.
32. Wyngaarden, J. B., H. R. Silberman and J. H. Sadler. *Ann. New York Acad. Sc.* 75: 45, 1958.
33. Koch, A.: In: *Symposium on Information Theory in Biology*, edited by H. P. Yockey. London: Pergamon, 1958, p. 136.

AMERICAN PHYSIOLOGICAL SOCIETY

Symposium on Life In Space[1]

Chairman: WALLACE O. FENN

INTRODUCTION

WALLACE O. FENN

University of Rochester Medical Center, Rochester, New York

THIS SYMPOSIUM is devoted to the physiological aspects of the new science of outer space. This at least is my interpretation of the title Life in Space which by itself does not have a very specific meaning. Perhaps a better title would have been 'Physiology in Orbit.' Regardless of the general title, however, the printed program outlines adequately the subject to be discussed.

I was pleased to accept the invitation of the Council to organize a symposium around this title. It appeared to me, however, that the real chairman of such a meeting should be someone who has himself contributed experimentally to the subject and in this respect I immediately thought of one member of our Society who is particularly distinguished in this respect. I made this suggestion to the Council and Major General Harry G. Armstrong, Surgeon General of the Air Force (1949–1954), was appointed Honorary Chairman. This appointment was graciously accepted by General Armstrong and it should be regarded as an indication of the desire of the Council to express to him the respect and admiration which the Society feels toward his accomplishments in the field of Aviation and Space Medicine. As Director of Research at the Aeromedical Laboratory at Wright Field from 1934–1940 he laid the foundations for this subject by his classical book on the Principles of Aviation Medicine, now in its third edition. At the same time he contributed much to the progress of our science by helping to establish the strong applied field of aviation physiology in which many of our members were active during the last war. Some ten years later, as Commandant of the School of Aviation Medicine at Randolph Field, General Armstrong established the Department of Space Medicine, the first in this country if not the first in the world. I had occasion to visit Randolph Field at that time and I remember that General Armstrong told me that this time the physiologists were going to be ahead of the engineers. When they were ready to put a man into space the physiologists would be ready to equip him. This year the department which he founded celebrated its tenth anniversary and we are fortunate today to have Colonel Campbell, the present experienced and able Chairman of that Department, as the closing speaker on this symposium. In establishing such a department General Armstrong demonstrated a great deal of wise foresight and much courage in the face of adverse criticism. How could he have foreseen that today, only ten years later, the world would have two satellites orbiting around the sun and many others in orbit around the earth? Today space science is the challenge of the hour and on account of the military potentialities it is sometimes regarded as a necessity for the defense of freedom in the world.

In inviting General Armstrong to be present in person at this meeting I reminded him that many of the younger physiologists had never even seen the man who contributed so much to our science and I assured him that a few words from him here would be much appreciated. I regret very much that he was not able to accept this invitation. It is a pleasure, nevertheless, to put on record this sincere tribute to his important accomplishments.

[1] Atlantic City, New Jersey, April 17, 1959.

RADIATION AND LIFE IN SPACE

CORNELIUS A. TOBIAS

Donner Laboratory, University of California, Berkeley, California

WE CANNOT imagine life on this earth without radiation. Biosynthesis of some essential organic molecules proceeds by photosynthesis, thus making visible radiation essential to life. The more penetrating natural radiations from the radioactivity of the earth and from cosmic radiation have been present since the beginnings of the solar system and appear to have a definite role in causing genetic variation. Thus radiation is a factor in evolution. Studying the Universe, we must perceive at once that conditions on the surface of the earth are very special indeed to allow physical, chemical and radiation environment so gentle and stable that in the span of perhaps less than two billion years a great manifoldness of living organisms has arisen.

As far as man can reach with the tools of astronomy, the Universe consists of highly dense matter in the stars which are in a state of thermonuclear interaction at many millions of degrees temperature. The space between the stars is a very high vacuum except where filled by cosmic gas, dust or ionized plasma. The entire spectrum of known (and perhaps unknown) electromagnetic and corpuscular radiations is present in space. At present it is believed that most of matter and radiation is within the galaxies, and if this assumption is true, then we know that the energy content of the Universe is mainly contained in the mass of the stars and to a very small extent in the cosmic radiation.

We receive a good deal of radiation from the sun ; part of the solar spectrum, visible light, is quite essential to life being the main energy source of photosynthesis. The visible infrared and ultraviolet spectrum also contribute to the surface and air temperature and keep these in narrow range. The sun appears to be a sphere of 5800° kelvin surface temperature having a spectrum of emission of electromagnetic waves near the theoretical 'black body' emission. Most of the ultraviolet and x-ray components are absorbed in the upper atmosphere, and the visible and infrared rays deposit an average of 2 cal/min cm^2 on the earth's surface (1). Deviations from the ideal 'black body' spectrum due to the higher temperatures of the solar interior, and the violent solar surface phenomena, have been demonstrated in measurements from high altitude balloons and rockets (2), and a summary of present-day knowledge is presented in figure 1. The most striking deviations are the emission lines from hydrogen in the solar atmosphere, the strongest of which is the Lyman alpha line in the far ultraviolet and the x-ray emission, coming in part from highly ionized atoms in the solar corona. During solar flares x-rays up to several million volts in energy reach the upper atmosphere, and the x-ray intensity at some wave lengths can be as much as 100,000 times the black body radiation.

The ultraviolet rays of the sun are in part responsible for dissociation of air molecules and the appearance of radicals of O, N, H and their products (3) above 50 km altitude. Lower, in the stratosphere, ozone gas is present which in turn absorbs near ultraviolet radiations, thus protecting life below. On the surface of the moon and on planet Mars the ultraviolet radiation could seriously influence the existence of living cells. It is in the region below 3000 A° where nucleic acids and protein absorbs strongest and where lethal and sterilizing effects of ultraviolet are greatest (4). Thus we can postulate that on Mars or the Moon living cells would have to be resistant to ultraviolet rays, possess protective coating or live in places sheltered from the direct rays. On the other hand, the surfaces of Venus and Jupiter appear to be well shielded from ultraviolet rays by their atmospheres.

Space ships can be shielded from ultraviolet rays and low energy x-rays; the more penetrating components of primary cosmic rays and their secondaries present a health problem. Actually the surface of the earth is quite well shielded from the more penetrating components of intragalactic radiation. The atmosphere, which absorbs most of the cosmic rays and the earth's magnetic field, deflects away most of the charged particles impinging on the surface. It appears that less than 2% of the primary cosmic ray intensity in space reaches us at ground level. These protective features are present to varying degrees on the other planets.

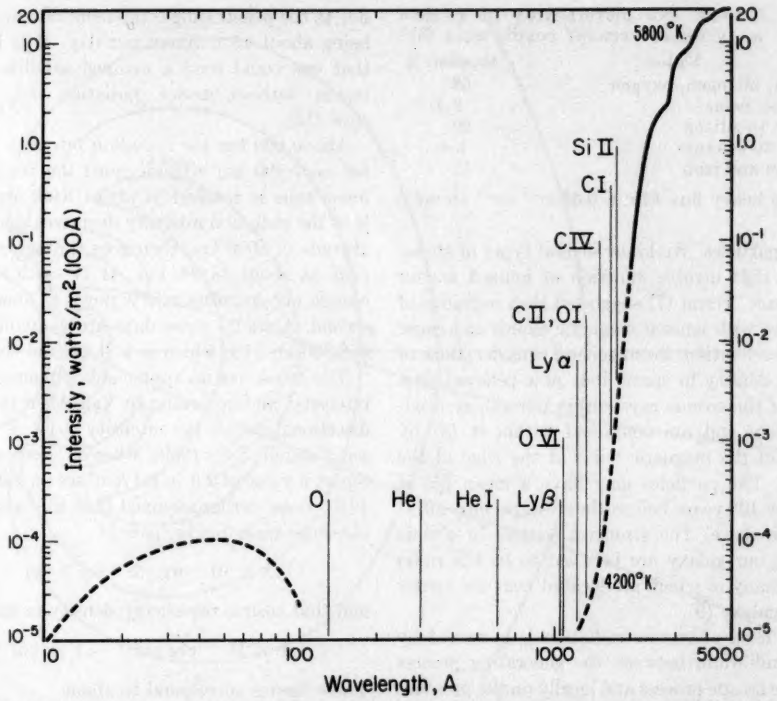


FIG. 1. Intensity of vertically incident solar radiation in spectral region below 5000 A°. In visible region sun appears to have surface temperature of about 5800 A°. This drops to about 4200 A° in the ultraviolet but several intense spectral lines of hydrogen and other elements have been observed in the far ultraviolet and soft x-ray region. X-ray region corresponds to a temperature of about 1,000,000°K and has great variations in intensity (2).

PRIMARY COSMIC RAYS

These are radiations which have their origin in extraterrestrial sources. Our present picture of the primary cosmic radiation is that of rapidly moving atomic nuclei, stripped of their electrons and converging incessantly on the earth homogenously from each direction in space (5). Protons are most abundant, and the frequency of heavier nuclei diminishes with increasing atomic number. In addition to the positively charged atomic nuclei electrons, fast neutrons, gamma rays and anti particles have been postulated as part of the primary rays, and we know that neutrinos are also present. Observation of the latter classes of primary particles has not as yet been successful because of their low abundance and the fact that such measurements need to be done away from the disturbing influence of the earth's atmosphere and magnetic field, at distances of more than 50,000 miles.

Near the earth a mixture of primary and secondary cosmic radiations is observed. Part of the primary flux of particles is reflected and another part captured by the earth's magnetic field thus causing a redistribution of particles. In the atmosphere by inelastic collisions the primary particles break up into a number of different kinds of secondaries.

Table 1 lists the frequency of various atomic nuclei in the primary cosmic radiation at rocket altitude. The heavy ion data were obtained by Yagoda (6) in high altitude rocket flights using photographic emulsions to record the tracks of the particles.

From the isotropic distribution of the primary rays, it seems clear that the majority of them must originate outside of the solar system, and in order to explain their presence one must propose a satisfactory injection and acceleration mechanism, as well as one for elimination of some

TABLE 1. FLUX AND DISTRIBUTION OF CHARGE AT 41° N. IN HEAVY PRIMARY COSMIC RAYS (6)

Nucleus	Abundance %
Carbon, nitrogen, oxygen	58
Fluorine, neon	9.1
Sodium to silicon	20
Silicon to calcium	low
Calcium and iron	11

Total heavy flux 4.98 ± 0.65 m^{-2} sec^{-1} $sterad^{-1}$

of the particles. We know several types of stellar events that involve emission of ionized matter into space. Fermi (7) suggested that collisions of particles with ionized magnetic clouds can cause their acceleration. From general considerations of energy density in space, it is now believed that most of the cosmic rays within our galaxy originate here and are contained within it (8) by virtue of the magnetic fields at the edge of the galaxy. The particles may have a mean life of perhaps 10^6 years before they escape into intergalactic space. The strongest sources of cosmic rays in our galaxy are believed to be the radio stars, many of which are located near the center of the galaxy (9).

The level of cosmic radiation is governed by the equilibrium between the generating process and the escape process and locally on the presence of magnetic activity resulting from cosmic clouds. The above outlined principles predict an energy distribution for the primaries in agreement with the experimentally found distribution at high energies per nucleon (above several Bev/nucleon). If $N(E)$ be the number of particles in energy range dE

$$\frac{dN}{dE} = -E^a \quad \text{where } a = -1.8$$

This relationship is followed up to the highest primary events: $E \sim 10^{16}$ ev.

RADIATION BELTS

With the first successful American satellite, Explorer I, regions of high intensity radiation around the equator were discovered, now bearing the name 'Van Allen radiation belts' after the leader of the research group which made the original observations (10).

Although much remains to be learned, we already have some detailed knowledge of the nature and origin of these rays, best summarized in Van Allen's own reports (11–14).

In Geiger Mueller counters up to 600 km the counting rate is low, and the radiation is chiefly due to the penetrating component of cosmic rays, being about 15 millirem per day. It is believed that one could send a manned satellite to this region without undue radiation risk to the crew (15).

Above 600 km the radiation intensity doubles for each 100 km altitude until the peak of the inner zone is reached at about 3400 km. From here the radiation intensity decreases again to an altitude of 8000 km, then goes through a second peak at about 18,000 km. At 17 earth radii the cosmic ray counting rate is down to about 2 per second. Actually some data are available up to 658,300 km (16) which is well beyond the moon.

The moon has no appreciable influence on the intensity, and according to Van Allen the omnidirectional cosmic ray intensity is 1.8 ± 0.3/cm^2 sec (March 3–6, 1959) whereas Vernov *et al.* report a value of 2.3 ± 0.1/cm^2 sec on January 2, 1959. Rossi earlier assumed that the energy flux of cosmic radiation in space is

$$3.5 \times 10^{-3} \text{ erg cm}^{-2} \text{ sec}^{-1} \text{ ster}^{-1}$$

and that cosmic ray energy density in space is

$$1.4 \times 10^{-12} \text{ erg cm}^{-3} \sim 1 \text{ ev cm}^{-3}.$$

These figures correspond to about

$$7.2 \text{ primary particles cm}^{-2} \text{ sec}^{-1}$$

and to a dose level of about 25 millirad per day.[1] The measurements are about 3 times lower than the last figure. Knowing that variations take place in the intensity, more data seem necessary to know the average level.

Figure 2 presents our present idea of the distribution of radiation in the two radiation belts. There have not been enough rocket flights to know the exact distribution, but the actual distribution is at least as complex as the one shown.

Even before cosmic rays were discovered the existence of radiation ring currents around the earth was postulated by Poincare, Stoermer and others to account for minor variations in the earth's magnetic field. For some years both experimental (17) and theoretical evidence was available to the effect that a magnetic field can trap charged particles injected at the proper angle and energy. The particles, once trapped, spiral around the magnetic lines of force. The shape of the earth's magnetic field is such that some of the particles are reflected when they get

[1] 1 rad = 100 ergs g^{-1}; 24-hour day.

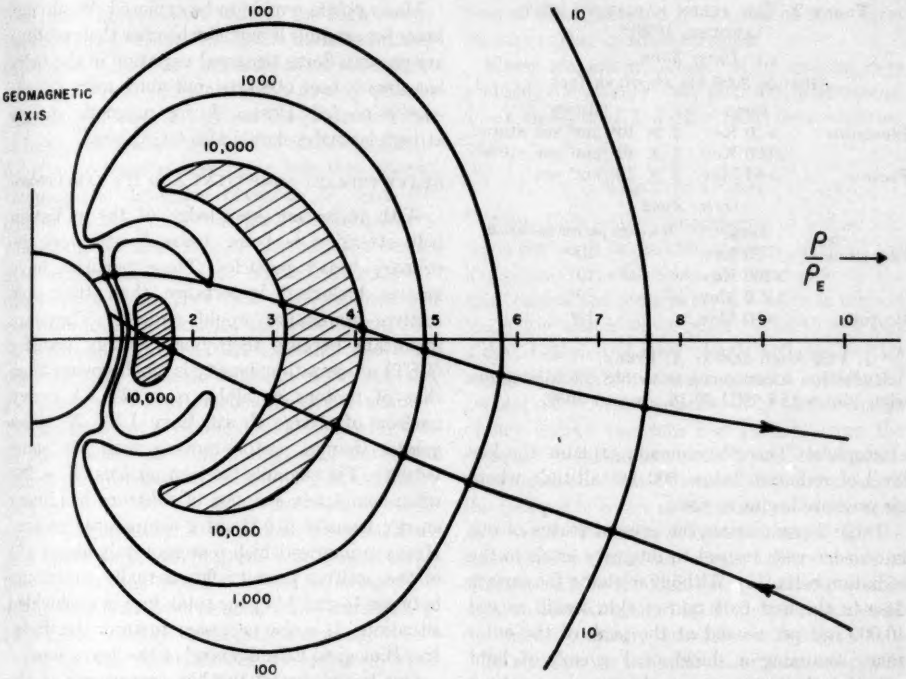


FIG. 2. A plot in a geomagnetic meridian plane of the intensity structure of radiation belts around the earth. Se is the earth's radius. Contour lines represent assumed extension of actual counting data which were obtained along the two lines indicated by arrows. Numbers associated with the several contours of constant intensity are the true counting rates in counts per sec. of a Geiger Mueller tube in Pioneer III or in satellite 1958E. From Allen (12).

near 40° latitude north or south. Moving in the earth's magnetic field it is possible that the particles will be accelerated also, due to gradients in the magnetic field.

The radiation intensity in the Van Allen band is high because particles are trapped in a finite region of space. It is believed that at least in the outer zones renewal of the electrons and some of the positive particles happens when the earth collides with an ionized magnetic cloud originating perhaps from the sun. The outer belt with its low magnetic field appears to be more efficient in retaining electrons than positive nucleons, and very few if any of the latter are present.

There is direct experimental proof based on tracks of particles on photographic emulsions (18) that the inner belt in addition to electrons contains protons up to 700 Mev kinetic energy. According to suggestions and theories by Christofilos (19), Singer (20) and others, these protons are the result of radioactive decay of neutrons which are produced by inelastic collisions of primary cosmic rays in the upper atmosphere. The neutrons decay into protons, electrons and neutrinos with a twelve minute half-life.

Loss of particles from both radiation belts can happen along the magnetic lines of force near the north and south poles, particularly during magnetic storms. It is believed that the particles thus lost from the radiation belt are at least in part responsible for the auroral phenomena.

The storing capacity of the radiation belt for charged particles has been directly demonstrated in the 'Argus' experiments originated by Christofilos (21) when small atom bombs were exploded between the two natural radiation belts. As observed in satellites and rockets (22), artificial radiation bands resulted. By such means it is possible to cause regions around the earth with considerable radiation intensities and persistence—a potential man-made hazard to space travel.

Particles are lost from the lower radiation belt also by scattering with the atoms of the earth's

TABLE 2. VAN ALLEN RADIATION BELTS (AUGUST, 1959)*

Inner Zone

Altitude 3600 km above surface

	Energy	Intensity
Electrons	>20 Kev	2×10^9/cm² sec sterad
	>600 Kev	1×10^7/cm² sec sterad
Protons	>40 Mev	2×10^4/cm² sec

Outer Zone

	Energy	Intensity per cm² sec sterad
Electrons	>20 Kev	10^{11}
	>200 Kev	10^8
	>2.5 Mev	10^6
Protons	>60 Mev	10^2

* J. Van Allen and L. A. Frank.
Radiation measurements to 658,300 kilometers with Pioneer IV SUI 59-18, August 1959.

atmosphere. This phenomenon explains the low level of radiation below 600 km altitude where air pressure begins to rise.

Table 2 summarizes the present status of our knowledge with respect to intensity levels in the radiation belts (14). Without shielding the surface dose in the first 0.01 mm of skin would exceed 40,000 rad per second at the peak of the outer zone. Assuming a shield of 1 g/cm², of light material, the inner zone would give rise to about 10 rad/hour, the outer zone in the order of 50 rad/hour. Because of the presence of high energy protons in the inner zone, shielding there seems impractical. In the outer belt most of the electrons are stopped by 1 g/cm² absorber and further shielding is chiefly a matter of absorbing the secondary x-rays. Their half thickness in lead is about 0.7 g/cm² so that 4 g/cm² reduces the radiation to one rad per hour.

It would then appear that the radiation belts limit the desirable space, in which manned space flight should be encountered, to certain regions:

1) Flight below 600 km altitude seems reasonably safe.

2) A space rocket may safely leave the earth in a narrow cone near the magnetic poles.

3) Shielding of the order of 10 g/cm² thickness removes most of the hazard from the outer radiation belt.

4) Shielding of the protons of the inner radiation belt seems impractical.

5) Beyond about 15 earth radii the effect of the radiation belts may be crossed.

6) The radiation belts may be crossed by a rapidly moving rocket at the expense of receiving a few rem dose.

Many points remain to be explored. We do not know for example if particles heavier than protons are present. Some temporal variation in the belts has already been observed, but much more knowledge is needed. Dosage during magnetic storms at high latitudes should also be explored.

HEAVY PRIMARY COMPONENT AND ITS VARIATIONS

With increasing knowledge of the radiation belt attention is being focused again on the primary heavy particles. These particles have greater biological importance than their low relative abundance would indicate. They are important because their linear energy transfer (LET) or ionization density is much greater than that of protons or alpha rays (23). A heavy nucleon of charge Z will have LET Z^2 times greater than a proton moving with the same velocity. For example, an iron nucleus ($Z = 26$) when completely stripped of electrons has linear energy transfer 676 times a comparable proton. Heavy primaries which represent only about 1% of the positive primary flux actually contribute between ½ and ⅓ of the total dose in unshielded situations. It is also necessary to know the 'Relative Biological Effectiveness' of the heavy ions.

Our knowledge of the low energy end of the primary heavy nucleon spectrum has been very limited. Due to the geomagnetic cutoff only nucleons higher than about 0.7 Bev per nucleon reach the top of the atmosphere in the temperate zones. Over the magnetic poles lower energy particles could come in, and their absence led to an early conclusion to the effect that primary rays usually would not include many particles of low energy. Study of the cosmic ray variations has, however, shown that there are many low energy particles generated by the sun. Such particles arrive with preference in the northern temperate zones. In order to completely assess the possible radiation hazard we must know the charge spectrum and energy distribution of heavy primaries to levels lower than 100 Mev per nucleon.

COSMIC RAY VARIATIONS

A number of important solar flares have been recorded during the past 15 years (24). During an important solar flare in 1956 (25) there was an increase of as much as 30-fold in neutron intensity near ground level (26) within 15 minutes, reflecting similar increases in the primary particles (at 54°N, 71°W) whereas mesons only increased 58% (27). The increase in the primary low energy component, up to 4 Bev/nucleon,

was much greater than that in the high energy part of the spectrum. One of the most interesting aspects of cosmic ray increases after solar flares is that the rays strike the earth in very uneven distribution. Firor calculated the probable zones of impact of cosmic rays on the earth's magnetic field; some zones receive more dose than others. Geomagnetic latitudes between 25° and 60° exhibit an increase, while the equatorial North American continent falls in the heaviest irradiated zone. Existing data on the geographical distribution of the flare-type increases bear out the theoretical predictions, and thus we are now quite certain that the particles arriving associated with the solar flares do originate in the sun and are accelerated in a reasonably direct manner. Extrapolation of available data led Schaefer (28) to the speculation that during solar flares the energy (E) distribution of heavy primaries might vary as E^{-6} or E^{-7}. If this were true, we could expect during flares 10,000 times the usual dose in a space ship with 1 g/cm^2 shielding. Taking into account the duration of the flare, a person might receive as much as 25 rad, and in unusually large flares possibly 10 times that amount.

During the International Geophysical Year about one flare a month has been reported. In order to know the flare radiation spectrum in detail it is necessary to send rockets and balloons to high altitude during various phases of each flare. Since we do not know of advance signs of an impending flare, constant optical observation of the sun is used. The first successful interception of a solar flare was in July, 1959. In this event it was reported (29) that the low energy component of primary cosmic radiation transiently increased by a very large factor. Measuring near the top of the atmosphere Brown (30) found for the energy interval 100 Mev $< E <$ 400 Mev that the number of protons increased like $E^{-4.5}$. It also seems reasonable that should one attempt to fly closer to the sun than we are at present, the cosmic rays of solar origin should increase in intensity at least as fast as the inverse square distance law would predict. Now a high primary radiation intensity in prolonged flight or in residence on one of the planets may make serious limitations on life span and evolutionary processes. Thus, solar cosmic rays should be considered in the definition and limitations of Strughold's Heliocosphere (31). Contributions of solar-flare doses of cosmic rays to the average dose level at ground level are not significant. The quantitative observations have been too recent, however, to predict what variation in the size of solar flares we may expect in times to come.

Flares are also accompanied by gamma rays probably secondary to nuclear interactions. Their energies of 3–5 Mev have been reported (30).

MAGNETIC STORMS

While high energy components of a flare can reach the earth in minutes or hours, most of the ion plasma expelled from the sun adds to the solar corona and some parts of it move in ionized 'magnetic' clouds which reach the earth in about a day. Collision of these clouds with the earth's magnetic field appears to cause a variety of events; one of these appears to be the discharge of low energy electrons and protons from the radiation belt near the polar zones with accompanying phenomena of aurora, redistribution of the Heavyside layers of electrons, radio blackout and world-wide meteorological changes. At the same time the magnetic clouds appear to act as effective shields for high energy galactic cosmic rays, so that at ground level the over-all cosmic ray intensity usually decreases for 1–3 days.

In addition to the solar flares, other types of solar events also influence cosmic ray intensity as reviewed by the author (32). Among these are variations with the 20-year solar cycle and a rather large variation with 11-year cycle corresponding to the frequency of sunspots.

THE BIOLOGICAL PROBLEM

If we are to insure radiation safety for man in rockets and satellites, there are important tasks remaining for the radiation physicist and biologist. Some of these will be listed briefly:

Dosimetry

In order to understand and measure the magnitude of biological hazard we must be able to measure the all essential factors. These include the measurement of the time rate of 'absorbed dose,' that is the energy transmitted to each unit mass of tissue per unit time. We should also know the nature, energy and spatial distribution of the radiations along with nuclear interactions they may cause in the atoms of tissue. The radiobiologist wishes to know the microscopic distribution of frequency of ionizing tracks with different ion density, that is the distribution of 'linear energy transfers.' Complete understanding of these factors requires development of special dosimeters. These should accompany biological

test object on satellite flights. Their presence seems essential for some purposes in view of the very large spatial and temporal variations in cosmic rays.

Shielding

It may be stated that man or most living organisms could not exist for long periods of time in extraterrestrial space without some radiation shielding. We already know that a reasonable shield (1 g/cm^2) will stop a good deal of radiation, and with increasing knowledge refined shields can be designed, even though some parts of space (e.g. the inner radiation belt) still remain hazardous.

Biological Effects of Heavy Ions

We do have actual and potential means to study effects of heavy cosmic ray primaries on accelerated beams at ground level. The effects of proton and alpha particle beams have been under study for some 10 years using cyclotrons (33) and two linear accelerators which are available for study of heavy ion effects. At the Berkeley HILAC machine carbon, oxygen, neon and argon beams are available with 10 Mev energy per nucleon and a similar machine is available at Yale University. During the past two years the effects of these beams on some unicellular organisms (34), phage and enzymes have been studied, and a beginning was made toward studies on animal tissues, particularly skin and brain. The range of these particles is very limited, however, (500 microns in tissue for carbon) so that one should try to accelerate heavy nuclei up to levels of 1 Bev per nucleon in order to observe their effects on the whole animal.

When compared to effects of widely used radiations, heavy ions impress with their ability to inflict great local damage, distributed in a statistical manner to locations near heavy ion tracks. It is believed that the greatest effect of such tracks is on cells essential to the whole organism, e.g. certain essential parts of the central nervous system (hypothalamus, brain stem, optic nerve) or on cells of the developing embryo.

When the influence of radiation upon extraterrestrial life is being considered, many challenging problems appear. Can a single organism or a population adapt themselves to a high radiation level? The theoretical answer suggests that the highest level is the one where complete reproduction is possible for many generations before the dose received becomes lethal to the parent or, by genetic changes, to the offspring. For some lower organisms, for example yeast cells (35), we have some idea about the magnitude of these levels. For mammals our knowledge is mostly by inference.

When man visits other planets of the solar system he may be confronted with primordial conditions on some planets, strange forms of life on others, and perhaps he will find planets where ancient life has become extinct. It seems to be of import to ascertain the role of penetrating and solar electromagnetic radiation to the generation of biological materials and to the mutation and evolution of the species. Great strides are being made in these fields (36, 37), still we must admit that we do not know the relative role of radiation as compared to chemical environment and temperature in evolution. The interplay of these factors during the last two billion years of our own history has been of decisive influence in bringing the living systems to their present state.

The fundamental problems just mentioned can be vigorously attacked in our earthbound laboratories. No matter how far we can carry such investigations, it seems nevertheless certain that space travel will bring further surprises and much new knowledge.

REFERENCES

1. Kopal, Z. *Brit. J. App. Phys.* 7: 119, 1956.
2. Watanabe, K. *Advances in Geophysics.* New York: Acad. Press, 1958 p. 153.
3. Kaplan, J. *The Chemosphere.* Second Internat. Symp. on the Physics and Medicine of the Atmosphere and Space, November, 1958. New York: Wiley. In press.
4. Hollaender, A. (editor). *Radiation Biology, Vol.* II, New York: McGraw-Hill, 1955.
5. Freier, Phyllis, E. J. Lofgren, E. P. Ney, F. Oppenheimer, H. L. Bradt and B. Peters. *Physiol. Rev.* 74: 213, 1948.
6. Yagoda, H. Geophysical Research Papers No. 60, ASTIA Document No. AD-152585, 1958.
7. Fermi, E. *Physiol. Rev.* 75: 1169, 1949.
8. DeHoffmann, F. and E. Teller. *Physiol. Rev.* 80: 692, 1951.
9. Unsold, A. *Zeitschrift für Astrophysik.* 26: 176, 1949.
10. Van Allen, J. A., G. H. Ludwig, E. C. Ray and C. E. McIlwain. *Jet Propulsion* 28: 588, 1958.
11. Van Allen, J. A., C. E. McIlwain and G. H. Ludwig. *J. Geophys. Research* 64: 271, 1959.
12. Van Allen, J. A. and L. A. Frank. *Nature, London* 183: 430, 1959.
13. Van Allen, J. A. and L. A. Frank. State

Univ. of Iowa Report SUI 59-18, August, 1959.
14. Van Allen, J. A. State Univ. of Iowa Report SUI 59-7, May, 1959.
15. Langham, W. H. Institute of Aeronautical Sciences Report No. 59-52.
16. Vernov, S. N., A. Y. Chudakov, P. V. Vakulov and Y. I. Logachev. Doklady Akad. Nauk SSSR 125: 304, 1959.
17. Chamberlain, J. W. *Advances in Geophysics*. New York: Acad. Press, 1958.
18. Freden, S. C. and S. White. Univ. of Calif. Lawrence Rad. Lab. Report UCRL 5581T, May, 1959.
19. Christofilos, N. C. State Univ. of Iowa Report SUI 59-16, 1959, p. 13.
20. Singer, S. F. *Phys. Rev. Letters* 1: 171, 1958.
21. Christofilos, N. C. Univ. of Calif. Lawrence Rad. Lab. Report UCRL 5548, 1959.
22. Van Allen, J. A., C. E. McIlwain and G. H. Ludwig. State Univ. of Iowa Report SUI 59-10, May, 1959.
23. Tobias, C. A. *J. Aviation Med.* 23: 345, 1952.
24. Elliott, H. In: J. G. Wilson's *Progress in Cosmic Ray Physics*. Amsterdam: North-Holland Publ. Co., 1952, Chap. vii.
25. Brode, R. B. and A. Goodwin, Jr. *Physiol. Rev.* 103: 377, 1956.
26. Lockwood, J. A., R. E. Yingst, A. R. Calawa and G. Sarmaniote. *Physiol. Rev.* 103: 247, 1954.
27. Firor, J. *Physiol. Rev.* 94: 1017, 1954.
28. Schaefer, Herman J. *Solar Influences on the Estra Atmospheric Radiation Field and their Radiobiological Implications*. Second Internat. Symp. on Physics and Medicine of the Atmosphere and Space, November, 1958. New York: Wiley. In press.
29. Ney E. P., J. R. Winckler and P. S. Frier. *Phys. Rev. Letters* 3: No. 4, Aug. 1959.
30. Brown, R. R. and D'Arcy. *Phys. Rev. Letters* 3: No. 8, Oct. 1959.
31. Strughold, H. *J. Aviation Med.* 26: 323, 1955.
32. Tobias, C. A. Univ. of Calif. Lawrence Rad. Lab. Report UCRL 8115, January, 1958.
33. Tobias, C. A. and T. Brustad. *Radiobiological Studies with Accelerated Heavy Ions*. Second Internat. Symp. on Physics and Medicine of the Atmosphere and Space, November, 1958. New York: Wiley. In press.
34. Born, J. L., *et al. Progress in Nuclear Energy, Series VII* 2: 184. New York: Pergamon, 1959.
35. Tobias, C. A., R. K. Mortimer, R. L. Gunther and G. P. Welch. *Proc. of the Second Geneva Atoms for Peace Conference*, Geneva: United Nations, 1958, vol. 22, p. 420.
36. Miller, S. L. and H. C. Urey. *Science* 130: 245, 1959.
37. Calvin, M. *Am. Scientist* 44: 248, 1956.

SOME METABOLIC PROBLEMS OF SPACE FLIGHT

Hans G. Clamann

Department of Physiology-Biophysics, School of Aviation Medicine (ATC), Brooks Air Force Base, Texas

Any definition of space flight requires a definition of space. Such a definition should be based on functional rather than on topographical factors (1). For technical as well as for biological purposes the beginning of space and of space flight may be placed at an a altude of about 90,000 feet or 17 mtude, the d air hes. At te¶is-n ilite1tisity of thas decreasd to about one peer cent of normal, not enough to supply air breathing power plants of aircraft with oxygen. The realm of the rocket motor with its own oxygen reserve begins. No longer can a compressor efficiently pressurize and ventilate a cabin with air from the outside. The passengers of a craft cruising at altitudes higher than 17 miles are confined to a completely sealed cabin.

Physiologically, such confinement creates in the first place the problem of maintaining suitable respiratory conditions within the cabin. For a trip exceeding a few hours up to a day, it becomes necessary to supply the passengers with water and food. With still further increasing exposure time, not only water vapor, carbon dioxide and any other noxious gases have to be removed, but also urine and feces.

So far such conditions seem to resemble those existing in submarines. But a submarine is much roomier than a space cabin, can jettison waste material, surface for air exchange and has little or no difficulties of temperature balance. Some special characteristics for a space vehicle are: a) extreme restriction for the payload in weight (more accurately: mass) and volume; b) extreme restriction in power requirements; and c) weightlessness.

The problems listed under a) and b) seem to be

TABLE 1. RELATIONSHIP BETWEEN EXHAUST GAS VELOCITY, FLIGHT VELOCITY AND MASS RATIO OF A SINGLE-STAGE ROCKET

$$V_{ROCKET} = V_{EXHAUST\ GAS} \times \ln \frac{\text{MASS OF ROCKET WITH FUEL}}{\text{MASS OF ROCKET AFTER BURNOUT}}$$

(MASS RATIO)

ROCKET VELOCITY NECESSARY FOR:		
ESCAPE VELOCITY	25,000 MILES/HOUR =	36,700 FT/SEC
ORBITAL VELOCITY (ALTITUDE 300 MILES)	18,000 MILES/HOUR =	26,400 FT/SEC

EXHAUST VELOCITIES	
LIQUID HYDROGEN LIQUID OXYGEN	10,800 FT/SEC
HYDRAZINE LIQUID OXYGEN	8,350 FT/SEC
ETHYL ALCOHOL LIQUID OXYGEN	7,800 FT/SEC

relative ones, relieved with the construction of larger, more powerful rockets. This is only partially true, since the relationship between weight of the payload and performance of a rocket is much closer than in any other vehicle. A short review of this weight-performance relationship, therefore, seems to be justified.

There are two especially significant flight conditions for rocketships: to orbit around the earth and to escape from the earth toward other planets. The velocity required to initiate orbital flight (at an altitude of 300 miles) is 18,000 mph, while the escape velocity amounts to 25,000 mph. (See table 1.) The velocity of a rocket (V_{rocket}) equals the velocity of the exhaust gas ($V_{exhaust}$) times a factor. This factor equals the natural logarithm (lr) of the so-called mass ratio. The mass ratio is the quotient of the total mass of the rocket with fuel before start over the mass of the rocket without fuel, that is, after all the fuel has been consumed during the first short powered phase of a rocket flight. From table 1 it can be seen that even the most powerful of all chemical combinations, liquid hydrogen plus liquid oxygen, would produce not even half the orbital velocity or one third of the escape velocity. In the latter case, the (lr) of the mass ratio should be about 3 or the mass ratio itself about 20. Figure 1 shows the ratio between the mass of the total rocket and the mass of the fuel as a curve. The upper abscissa represents mass ratios, the lower abscissa exhaust velocities. Both abscissas are plotted against an ordinate which is calibrated in per cent of the total rocket mass being 100%. The curve demonstrates how rapidly the percentage of fuel mass increases with increasing mass ratio. For a mass ratio of 20, for example, the weight of a rocket structure including any payload is 5%, the weight of fuel 95% of the total weight.

The mass ratio can be improved by using multi-stage rockets. In this case, not only fuel, but also part of the rocket structure itself is 'lost' during the powered phase of flight. The final stage then has to accelerate only a fraction of the original mass. But as pointed out before, the payload will be only a small fraction of the rocket. Even a future gigantic rocket, such as the five-stage 'Nova' with a total weight of 4,500,000 pounds, can carry to Mars only several tons of payload (2). Thus the weight-ratio payload/rocket would be of the order of one/one thousand.

For the first short trips into space, such as circumnavigation of the earth, the supply problem is not an urgent one. Questions of safety are more important. These include avoiding leakage or even rapid decompression of the capsule, and safe re-entry into the atmosphere through a period of high temperature of friction and finally safe landing.

With increasing time for space trips, more weight of supply is needed. Two conflicting problems have to be solved: to maintain optimum environmental and metabolic conditions required for full physical and mental performance of the space crew and to find the minimum of supply for this purpose.

To reduce the weight of supply needed for each passenger two main theoretical possibilities exist.

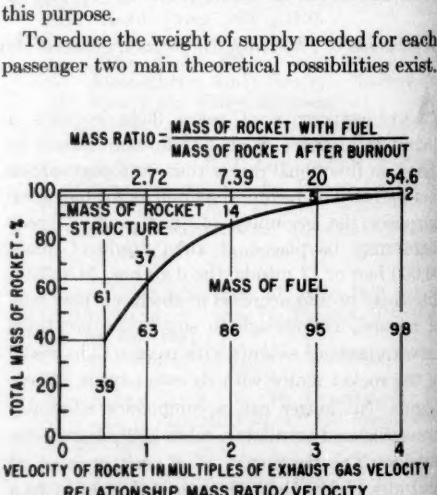


FIG. 1. Ratio of the total mass of a rocket vs. the mass of fuel.

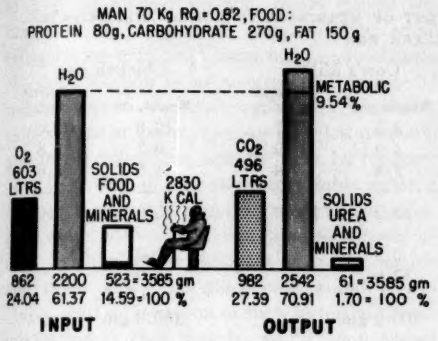


FIG. 2. Daily metabolic turnover of an adult person at light work.

The first of these possibilities is of a biological nature: to find the minimum of food to yield the maximum of energy for the planned state of physical activity; simultaneously such diet should require a minimum consumption of oxygen, water and minerals, and have a minimum of indigestible residue. The second possibility, a technical one, lies in the utilization of recovering, regenerating and recycling processes. Such methods gain in importance with increasing duration of any space trip and will eventually become mandatory.

The energy requirements of an adult person depend primarily upon his muscular activity. While such activity may vary with the type of space craft and kind of expedition, it may be safe to assume at present an activity corresponding to that of an airplane pilot. Based upon such activity, a daily metabolic turnover of 2800 Cal. is anticipated (3).

An interesting question is: would weightlessness cause any decrease in the metabolic rate due to an assumed decreasing activity of muscles normally active in balancing the body in the gravitational field of the earth? Independent of gravitational forces, muscles have to be accelerated and decelerated in any movement. In terms of physics, muscles perform, by virtue of synergistic groups of muscles coordinated by reflexes, critically damped movements. In such a case, the resulting work equals $m \times v$, where m is mass and v is velocity. At the beginning of exposure to weightlessness the metabolic rate may even increase, before the muscles are adapted to the new situation.

Another interesting fact is that under the conditions as shown in figure 2, man's total output of water exceeds the input by approximately 10% and more per day. This excess is simply the metabolic water produced by oxidation of food. A further increase of fat in the diet would even increase the amount of metabolic water.

The inseparable relationship between metabolism and respiration is a fact often not fully recognized in studies on a closed environment such as the sealed cabin. For instance, very often it is recommended to store the food for a space trip in dehydrated form. This actually requires the recovery of at least so much from the excreted water as needed to satisfy the water input requirement. Without such recovery, storage of dehydrated food instead of food containing a large percentage of water would necessitate carrying just so much more water than extracted originally from the dehydrated food. Under circumstances otherwise alike, the quantity of water needed for metabolic purposes remains the same when offered as a beverage or as part of any food. Volume-wise separate storage of water and dehydrated food would even require more space than food combined with water since most dehydrated food has a density around 0.5 gm per cc.

It is well known that a diet containing protein, carbohydrates and fat in a proportion approximately as indicated is most suitable for the healthy adult. While the proportions of carbohydrates and fat can be altered, a minimum of protein as sole source of nitrogen has to be maintained. Figure 3 shows the quantity of carbohydrates, fat and protein, each in excess of a minimum of protein of 70 gm, necessary to produce 2800 Cal. The quantity of oxygen necessary

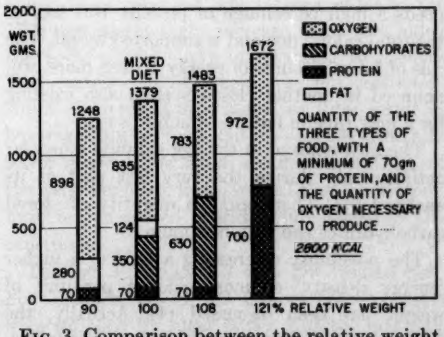


FIG. 3. Comparison between the relative weight of various combinations of nutrients plus oxygen. *Note:* height of last column indicates 770 gm protein instead of a correct height of 700 gm. Thus the total height of the column is 70 gm too high.

TABLE 2. COMPARISON OF THE RELATIVE CONTENT OF NUTRIENTS IN HUMAN MILK, COW'S MILK AND CHICKEN EGG

	Human Milk		Cow's Milk		Chicken Egg	
	Weight, gm	Caloric value, %	Weight, gm	Caloric value, %	Weight, gm	Caloric value, %
Protein	1.6	9.5	3.5	20.7	12.8	32.6
Carbohydrates	6.8	39.9	4.9	28.9	0.7	1.8
Fat	3.8	50.6	3.8	50.4	11.5	65.6
Total	12.2	100.0%	12.2	100.0%	25.0	100.0%
Minerals	0.2		0.8		1.0	
Water	87.6		87.0		74.0	
	100.0 gm		100.0 gm		100.0 gm	

for oxidation is included in the total quantity. While it may be expected that fat with 9 Cal. per gm results in the smallest total quantity of supply, that is 10% less than a mixed diet as source of energy set as 100%, the fact that the weight of oxygen in this case is more than twice as large as the weight of food may be surprising. This diagram demonstrates clearly how important it is to consider dietary and respiratory requirements as a unit, if saving in weight of the total supply is the problem.

Saving in weight suggests also a high percentage of fat in the diet. Simple storage of food, without any recovery or regeneration, and neglecting problems of taste would thus result in the development of a soup-like food with a high content of fat. A uniform food of such kind, still in use and satisfying all metabolic requirements has been developed by nature 40 million years before space flight—milk (table 2).

The composition of milk, fairly uniform for all mammals, shows a high percentage of fat and protein. Of course a baby animal has to grow and needs a high percentage of protein. But half of the total caloric demand is supported by fat. The role of fat as a source of energy is even more pronounced in another closed system also existing for a long time in nature—the bird's egg.

The bird embryo in the egg exhibits muscular activity only during the very last part of its enclosed life. Therefore, the quantity of stored carbohydrates can be a minimum.

The possibility of creating a food of a higher 'energy density' or more calories per unit of weight has been discussed (4). Actually, the caloric value of some representatives of our normal food stands a comparison with an industrial fuel such as benzene quite well (table 3). A fatty acid (palmitic acid) has 9.34 Cal. per gm, while benzene has 10 Cal. per gm. Some substances with a fairly high caloric value, glycerine and ethyl alcohol can be tolerated in very small quantities only.

Another possibility would be to create food of higher caloric value by extending the chain of carbon atoms of some known nutrients. Taking the saturated fatty acids as an example, the basic law of the linear increase of such properties as heat of combustion with the addition of each carbon atom in the carbon chain is evident (table 4). Unfortunately, this gain is very modest: approximately 310 cal. per mole with each added carbon atom. Per gm of substance, the gain in heat of combustion for 12 carbon atoms from capric to behenic acid amounts to only 9.74 – 8.44 = 1.30 Cal. or about 15.4%, while the molecular weight increases 100%. The organism may even be unable, to develop or adapt special enzymes (5) to attack such artificially created substances. As an end result, the possibility to produce a nutritive tablet even a hundred times the size of an aspirin seems very remote.

So far only the first possibility of reducing the weight of supply by reducing the quantity of food and oxygen has been considered. How about the second possibility of recovery of water, regenerating of absorbents and a more or less complete recycling process?

One possibility has already been discussed: the recovery of water. For the first short orbital flights absorption of water and carbon dioxide by chemical absorbents will suffice. For future space trips of the order of a month or longer the quantity of necessary absorbents becomes not only prohibitive, but precludes the re-use of water. Once the possibility of regenerating any absorbents by heat is considered, this means the availability of some source of energy. A discussion, if such source consisted of utilization of sunlight or

of a small nuclear reactor, would exceed the limits of this paper. A comparatively small refrigeration unit could carry out simultaneously the condensation of all expired and transpired water, support condensation of water in distillation of urine at low temperature and low pressure, and freeze out carbon dioxide. Thus, by recovery of at least 90% of all water into potable water, a comparatively small supply of water at the beginning would suffice and dehydrated food could be used. With effective freeze-out of carbon dioxide and other gases, no absorbents would be required. But a discussion of such technical details interesting as they may be would again exceed the limits and the scope of this symposium.

Returning to metabolic problems, a simple pilot experiment shall be discussed. This experiment had the following purpose:

Three human subjects, one 56 years old and occupied mostly with laboratory and office work, and two young students of 19 and 16 years, attending college and high school, respectively, were fed about three weeks with a food willingly chosen to be unattractive and monotonous. Such a situation may well simulate or even exceed, both the activity as well as the nutritional restriction of a future space pilot of a large space vehicle with opportunity of some physical exercise. By using a pedometer, an average daily total walking mileage between 1.3 and 3.6 miles was measured. Further physical exercise was restricted to occasional light sport (tennis) or light garden work (lawn mowing).

The general requirements were as follows: *a*) only dehydrated, commercially available food was used; *b*) this food did not require refrigeration for conservation; *c*) most of the food had, at the moment of its use, been on the shelf over a year or longer; *d*) the food was paste-like or liquid so it could be fed by squeeze bottles in weightless state. Such requirements were fulfilled by using rolled oats, wheat germ, dried egg powder, dry skimmed milk, whole milk powder, sugar and vegetable fat. These ingredients were mixed with water, shortly boiled and served three times daily. As additional source of protein, dried beef was supplied; as additional source of fat, various roasted nuts and chocolate; dried apricots served as source of vitamin A; vitamin C was supplied in tablets. Water, and water with dry milk powder and coffee, served as beverages.

TABLE 3. HEAT OF COMBUSTION OF VARIOUS NUTRIENTS AND OTHER ORGANIC SUBSTANCES

Substance *1 gm*	Formula	Heat of combustion *Cal.*
d-Glukose	$C_6H_{12}O_6$	3.71
Sucrose	$C_{12}H_{22}O_{11}$	3.95
Starch	$(C_6H_{10}O_5)_n$	4.18
Palmitic acid	$C_{16}H_{32}O_2$	9.34
Glycerin	$C_3H_5(OH)_3$	4.31
Ethyl alcohol	C_2H_5OH	7.11
Benzene	C_6H_6	10.0
Hydrogen	H_2	33.90

TABLE 4. HEAT OF COMBUSTION OF SOME SATURATED FATTY ACIDS WITH AN INCREASING NUMBER OF CARBON ATOMS SATURATED FATTY ACIDS

Gen. Formula: $C_nH_{2n}O_2$

Name	Carbon atoms	Mol. wt.	Heat of combustion (*Cal.*) Per mole.	diff.	per gm
Capric	10	172.2	1453.6		8.44
				310.4	
Lauric	12	200.2	1764.0		8.81
				311.0	
Myristic	14	228.2	2075.0		9.09
				310.3	
Palmitic	16	256.3	2385.3		9.31
				310.5	
Stearic	18	284.3	2695.8		9.48
				617.3	
Arachic	20	312.5			
Behenic	22	340.4	3313.1		9.74

The total amount of calories as well as the percentage of calories supplied by the three types of food were not constant during the 23 days of the experiment.

Figure 4 shows some of the results on the oldest subject. The lower graph represents the quantity of calories supplied by protein, fat and carbohydrates as well as the total calories. The curve at the top of the diagram represents the body weight in kg. With only 2000 to 2800 cal. the body weight drops steadily from its original value before the experiment. The increase of fat on the 17th day and a slight increase in protein aided the body weight curve in returning to normal. The quantity of beverages was not restricted. Input of water is determined as total water of beverages and of food, output only as urine and water in feces, the latter assumed to be 75% of the weight of feces. Both, water balance and feces, were not measured over the full length of the experiment.

The diet was regarded as a low residue diet. For instance, up to 100 Cal. of carbohydrates were supplied by sugar cubes. But the quantity of feces was practically the same as compared to the quantity after the experiment, shown only for a few days in this diagram. A certain periodic-

ity in the quantity of the feces seems to exist, the small 'd' denoting diarrhic consistency. A study of the bacterial flora was not carried out.

As to the monotony, the food was well tolerated for about 2 weeks by all three subjects. Then a sudden antipathy developed for 2 days. Having overcome this antipathy, the food was eaten in a routine manner. A monotonous food can possibly be well tolerated for an extended time, if within certain intervals tasty morsels of subjective preference are offered. The role of psychological factors in eating is well recognized and may justify special attention for space conditions, as it has under flying conditions (6).

The total quantity of dehydrated food per day was about one pound (454 gm), the total quantity of water about 2000 gm, ⅓ of which was excreted as urine and in feces. With an additional quantity of 870 gm of gaseous oxygen, chemical absorbents for exhaled and perspired water the total quantity of supply may amount to about 10 kg or 22 pounds per day per person. With partial recycling of water (the metabolic surplus just compensating the losses) and physical removal of CO_2 this quantity will decrease to only 1.6 kg or 3.5 pounds per day per person (fig. 5). Two

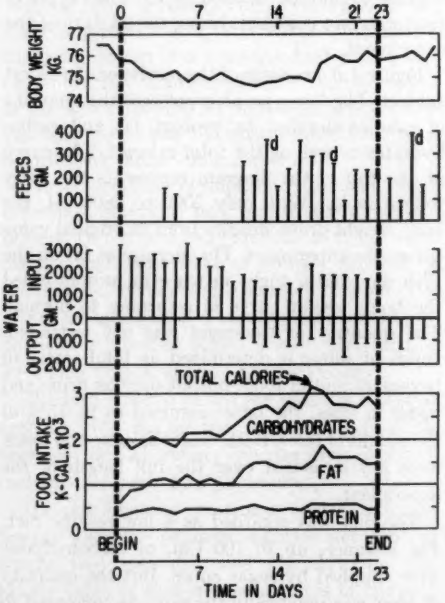


FIG. 4. Results of feeding a man for about 3 wk. with a prototype of poor 'space-food'.

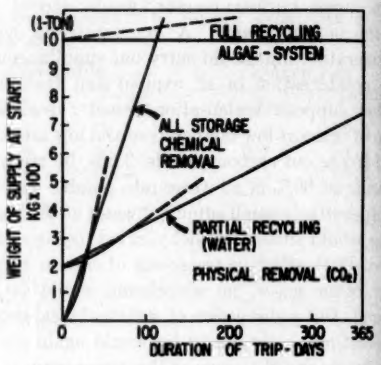


FIG. 5. Weight of supply for one person in a closed system (space ship), compared for 3 different systems with increasing time. (The dotted lines indicate the additional weight of spare parts.)

hundred kg or 440 pounds are assumed for the refrigeration unit and a small quantity of water to start. While this quantity is debatable, the slope of the curve would remain the same. Under the conditions as described, the water recycling, physical absorption system would be superior to the storage system with chemical absorbents within 3 weeks. An algae system may be feasible, if the duration of a space trip exceeds more than 1½ year.

The final and ideal goal would be a total recycling process of O_2, H_2O, and N_2 based upon the use of green plants (algae). While reconversion of CO_2 to O_2—at least theoretically—can be accomplished without biological means, the reconversion of carbon and nitrogen to food without the help of green plants seems even more remote. But even for an algae-system a host of problems remains to be attacked (7). The problem of illumination and of gas circulation under weightlessness has not yet been solved. A quantitative equilibrium of all metabolic requirements of man is not easily secured. Processing of the excess of the growing plant into a variety of food will require a considerable apparatus. Thus, a weight of one ton, quite in contrast to many authors, has been assumed for one person. This weight may remain fairly constant during a space trip, except for spare parts as indicated by the dotted line.

Rarely in all history of mankind has the biologist been challenged to such a degree as by the problems of space flight.

REFERENCES

1. Strughold, H., H. Haber, K. Buettner and F. Haber. *J. Aviation Med.* 22: 342, 1951.
2. NASA's New Rocket Booster Programme. *Interavia* XIV: 738, No. 6, 1959.
3. Tiller, P. R. *J. Aviation Med.* 28: 27, 1957.
4. Taylor, A. A. *Food Technol.* 12: 442, 1958.
5. Knox, W. E., V. H. Auerbach and E. C. C. Lin. *Physiol. Rev.* 36: 164, 1956.
6. Van Sickle, A. W. and R. J. Hogan. *J. Aviation Med.* 25: 523, 1954.
7. Clamann, H. G. *High Altitude and Satellite Rockets* (A Symposium held at Cranfield, England, 18–20th July 1957.) London: The Royal Aeronautical Society and The British Interplanetary Society, 1958, p. 102.

PROGRESS TOWARD SPACE FLIGHT[1]

Paul A. Campbell

U.S.A.F. Aerospase Medical Center, Brooks Air Force Base, Texas

Those of us associated with research toward the goal of manned space flight feel that its eventual accomplishment is inevitable, and that its accomplishment is a logical, rational development in the evolution of man and the evolution of the metagalactic universe.

Man has certain attributes, physiological, psychological and sociological, which have resulted in rational, step-by-step progress toward space. A few of these are: *a*) his natural curiosity which causes him to ask what lies beyond the clouds, the blue sky and the stars; *b*) the spirit of adventure from which he may gain enjoyment in going places and doing things outside the ordinary; in many cases he enjoys sufficient danger to separate him from the 'meek who shall inherit the earth'; *c*) his refusal to be contained by barriers which restrict him or his activities; *d*) his quest for achievement of which he, his family, etc. can be proud, which again sets him apart from his fellowmen and improves his ego; *e*) his built-in desire to do that which he has been told is impossible. How far man will go into space probably (and here we have all learned to couch our dogmatism with the term, probably) will be found to be limited to some extent at least by: *1*) the speed of light; *2*) the distance which he can travel and return, in his productive life span; unless he changes considerably he will always wish to return to tell or write about his feats; *3*) the limits of re-supply within reasonable time; *4*) the amount of radiation which he can be exposed to and remain in good health; and *5*) the statistical chances of survival.

This is all well and good and gives us a framework for the future and feeds fuel to the science fiction writers, but the cold gray dawn of each morning tells us that there is much to be done before space flight in its broader sense can be accomplished. We are progressing in a step-by-step fashion, but at times two steps forward and one step backward. Our forward steps sometimes bring us face to face with a new barrier, such as the Van Allen (1) type radiation bands, but new knowledge tempers the old and progress continues. In the time of those participating in this symposium, we have seen the oxygen barrier, the bends barrier, the vapor pressure barrier, the sound barrier, the thermal barrier, the ozone barrier, and many others, appear for a time to block extension of aviation, but each has vanished as some new breakthrough has shown the means of traverse. Progress in each parameter has been exponential. The pauses produced by barriers have resulted in such short lived plateaus that when viewed in the curve of progress of the Twentieth Century—59 years to date—they seem almost imperceptible. Aviation through rocketry, its Newtonian principle of propulsion, its lift produced by propellant rather than wings, and its ability to carry its oxidizer rather than to depend upon the atmosphere for its breath, now gives us the means for penetrating the atmospheric barrier. Where machines can go man wishes to go and will discover, invent or improvise the means.

But again to get back to our earthbound laboratories and our mundane existence, let us

[1] The contents of this manuscript reflect the personal views of the author and are not to be construed as a statement of official Air Force policy.

have a look at where we stand today, and point out a few of the more serious problems which slow progress toward space flight and require integrated, concentrated effort. Let us do a little curve watching before we look into the crystal ball.

Man and machine have been in a more or less continuous race to outdo one another since the advent of aviation. At times man, through advancements of the state of the art of protective devices and measures, has been in the lead and could go where the machine could not take him. At times the machine has been in the lead and has been able to go places and do things in which man could not participate. Until two or three years ago the race has been nip and tuck between the aviation designers and engineers on the one hand and the flight surgeons, aviation biologists and human factors groups on the other hand. But in these past two or three years the situation has suddenly changed and the machine capability has advanced far beyond man's capability. Let us look at two parameters to see where we stand and see this situation.

Manned altitude, or, as we must now say, distance-outward achievement has been one parameter which has been carefully watched since the Wright brothers' first flight. Let us have a quick look at it (fig. 1) because the curve is exponential and looks good when viewed on the proper chronological base line. It ended with Kinchloe's flight to an altitude of 126,200 feet. But when placed within the framework of hardware achievement as in figure 2 it does not look

FIG. 1. Aircraft alt. achievements plotted chronologically. Right-hand scale represents % of mass of atm. penetrated. Final point on graph is Kinchloe's record of 126,200 ft.

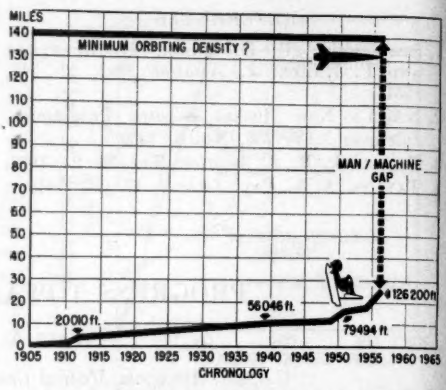


FIG. 2. Altitude achievements shown in fig. 1 plotted within framework of minimum orbital alt. to demonstrate the discrepancy between manned vehicle achievements and orbiting requirements.

so good. The man/machine gap is tremendous and is lengthening by the month.

Let us now look at another parameter—that of speed—because as we all know man cannot orbit until he reaches a speed of some 18,000 miles per hour and cannot escape the earth's gravitational tentacles until his velocity has reached some 25,000 mph. First, let us examine man's speed achievements plotted chronologically (fig. 3). It ends with Mel Apt's fatal flight reaching about 2148 mph. Again we have an exponential curve and man appears to be doing just fine. But (fig. 4) again, when viewed within the framework of hardware achievement, it does not look very good, and again we see a tremendous man/machine gap.

Now why has this gap lengthened so much in the past few years. I think we can point to one situation—a comparison of resources—scientists and facilities—a comparison between the hardware development area and the human factor development area. Whereas there are several thousand scientists and many facilities in hardware research, design and production there are still only a few in space medicine, space biology, human factors and related disciplines, and this is taking its toll in terms of integrated progress toward manned space flight.

The space concept is a relatively new one and is interdisciplinary throughout. Consequently, training for those who wish to participate simply does not exist except in the in-house, or on-the-job, categories. Programs for training require the

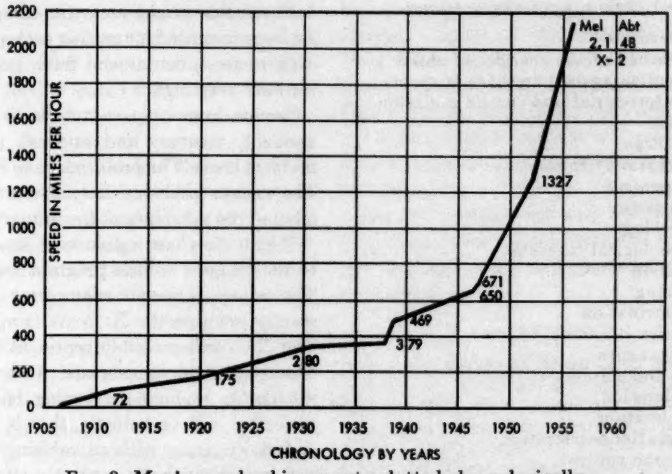


FIG. 3. Man's speed achievements plotted chronologically.

wedding of diverse disciplines such as astronomy and biology, astrophysics and ecology, logistics and ecology. As an example, may I point to the organizational chart of our own Space Medicine Division at the School of Aviation Medicine, USAF. (see table I). The wedding of the disciplines here is self evident

May I now emphasize that the primary biological problem of manned space flight at this time lies in the selection of people in the required interdisciplinary techniques and with imagination, who in turn can help produce solutions to the many complex problems which plague us.

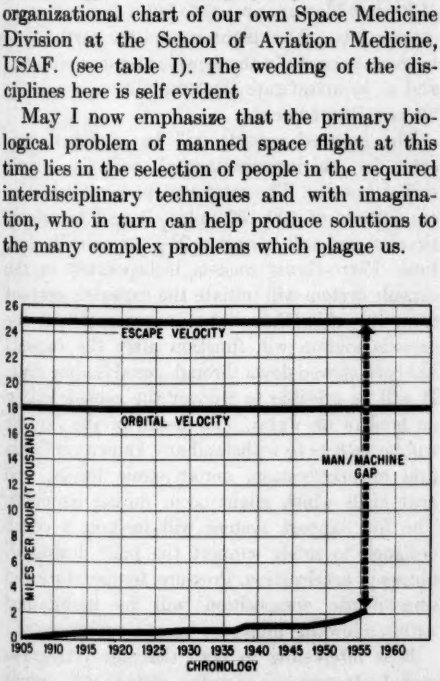


FIG. 4. Speed achievements shown in fig. 3 but plotted within framework of orbital and escape velocities to show discrepancy between man's attainments and machine capability.

Let us now have a look at some of the other problems in the production of a reliable manned space system and see where we stand today

The problem of re-entry into the earth's orbit is very serious as it involves relatively rapid slowdown from speeds (in the neighborhood of 18,000 mph if orbiting or some 25,000 mph if in escape ellipse) to zero mph. If we take the example of the orbiting vehicle, the magnitude (2) of the total energies is some 19 million foot pounds per pound of orbiting mass. Thus, approximately 24,000 btu's per pound of orbiting mass must be dissipated in a relatively short period of time. For comparison, the energy contained in gasoline is about 21,000 btu's per pound. During the same period high 'g' loads approaching man's tolerance limits will have to be sustained. Project Mercury, the first orbiting manned space craft, will have to meet these requirements. Their engineers say it can be done.

RADIATION

Radiation has been covered by a preceding speaker. I think he will agree that orbiting beneath the Van Allen (1) bands, yet remaining above the levels of appreciable atmospheric drag, is possible. This requires an almost circul r orbit between the altitudes of something like 140 miles and 400 miles. For travel into the deeper reaches of space, polar launching, to avoid the Van Allen bands, would require an exit passage almost identical with the path or entrance corridor of the maximum concentration of incoming

TABLE 1. SPACE MEDICINE DIVISION

1. *Bioastronautics*
   - Utilization of the energies of space
   - Protection against energies of space
   - Extraterrestrial and cosmic radiation
   - Liaison
2. *Astroecology*
   - Ecological systems
   - Components
   - Ecologistics
   - Synecology
   - Psychological reactions
   - Selection
   - Training
   - Indoctrination
3. *Biogravics*
   - Biodynamics
   - Zero and sub 'G'
   - Acceleration
   - Deceleration
   - Tangential acceleration
   - Reaction control
4. *Bioastrophysics*
   - Instrumentation
   - Design
   - Maintenance
   - Weight and capacity reduction
   - Re-entry

heavy primaries. Again in the type of space travel of the relatively near future, orbiting within these bounds for short periods seems feasible.

WEIGHTLESSNESS

This is another huge question as our simulation capability through the use of parabolic flight patterns still remains under something of the order of 60 seconds. We cannot even guess as to the effects of several hours or several days of zero 'g'. Several of us are of the opinion, however, that an even greater problem is for the engineers to provide an absolutely stable platform which will not produce some tangential 'g' due to rotation or tumbling. Weightlessness is possibly the lesser of the two evils.

CLOSED ECOLOGICAL SYSTEMS

For space travel of short duration such as a few circuits of the earth, closed loop ecological systems are unnecessary but for long flights involving months, such a system is an absolute requirement as resupply will be very difficult and the logistics will be exorbitant.

Human logistics of space flight (3) other than re-supply require capacity reduction through miniaturization, the conservation of everything, recycling and re-utilization wherever possible, etc. There is much to be done here and it must be done, as the weight costs (in terms of fuel and structure required for getting each pound of man, oxygen, food, containers, fluid, protective gear, etc.) are very high.

Escape from a space vehicle in the event of accident, recovery and survival, presents huge problem areas. The problems have been analyzed. The answer probably lies in constant improvement of the reliability of the primary vehicle.

Where does our nation now stand in relation to the manned vehicle program for space flight? The newspapers and engineering journals constantly mention the X-15 and Project Mercury. The X-15 will probably represent the transition of aircraft into space craft. According to news reports its preliminary testing has started. Its trajectory will be ballistic, that is up into space and down again without orbiting the earth. It will furnish much data as to the effects of weightlessness, the problems of re-entry, etc.

Project Mercury will be a manned orbiting vehicle. According to a recently released NASA Fact Sheet (4), the objectives and salient features of Project Mercury are: *a*) to put a manned space capsule into orbital flight around the earth; *b*) to recover successfully the capsule and its occupant; and *c*) to investigate the capabilities of man in his new environment.

The manned capsule will be launched into orbit by an intercontinental ballistic missile rocket booster. The orbit will be established at an altitude of 100–150 miles. It will be nearly circular and will permit a 24-hour satellite lifetime. Retro-thrust rockets incorporated in the capsule system will initiate the capsule's descent from its orbit. Parachutes incorporated in the capsule system will function after the capsule has been slowed down through aerodynamic drag. It will be possible to recover the capsule either on land or on water. The design of the capsule will be such as to withstand any known combination of acceleration, aerodynamic forces, and heat loads which might occur during its flight. The life support system will include a couch designed to safely support the pilot during all phases of acceleration. Pressure, temperature and atmospheric composition will be maintained within allowable limits for human environment.

It is interesting to note that the retrograde rocket atmospheric re-entry system will permit entry into the atmosphere in less than one-half an orbital revolution after initiation. The firing of the retro-rockets initiating re-entry can occur either from a signal, from a command link on the

ground, or by the man occupant, himself. The impact area can be determined through control of the point of re-entry into the atmosphere. The NASA release further stated that during re-entry and when the speed of the capsule has slowed to approximately the speed of sound, a drogue parachute will open to stabilize the vehicle. At that time, the release of radar chaff will pinpoint the capsule's location. A landing parachute will protect the capsule during the final stages of descent. From the standpoint of escape the requirements call for a method by which the capsule may be separated from the booster in case of emergency before orbital altitude is reached. During the orbital phase the atmosphere can be re-entered at the time the pilot may choose by activating the retro-rockets.

Of interest to this group, the capsule will be instrumented for evaluation of the pilot's reaction to space flight. Further instrumentation will be used to measure and monitor internal and external aspects of the capsule environment. Data will be recorded as well as telemetered. The final statement of the NASA release concerns the Test Program, and states that orbital flight of the manned space capsule will take place only after a logical build-up of vehicle capabilities and scientific data.

Our entire nation will wish the National Aeronautics and Space Administration complete success in this, a great steppingstone toward space exploration.

REFERENCES

1. Van Allen, J. A. *Scient. Am.* 200: 3, 1959.
2. Ross, F. W. *Space/Aeronautics*, 1959, p. 180.
3. Campbell, P. A. Cited in: *Vistas in Astronautics*, edited by Morton Alperin, *et al.* New York: Pergamon, 1958.
4. Project Mercury Fact Sheet. National Aeronautics and Space Administration, Washington, D. C. January 12, 1959.

INDEX TO VOLUME 18[1]

Abstracts of papers, 43rd Annual Meeting
Biochemistry, 177
Immunology, 555
Nutrition, 515
Pathology, 465
Pharmacology, 361
Physiology, 1
Amines, pharmacologically active, enzymic oxidation of, 1005
Amino acid, balance and protein requirement, 1136
Annual Meetings
Atlantic City (1959), 607
Chicago (1960), lvi
Autonomic pharmacology, symposium, 1001–1012

Bacterial cell walls, chemistry, 966
Biochemical aspects of cell structure and function, symposium, 957
Biochemistry, topics of current interest, symposium, 966–1000
Biological Chemists, Society of
abstracts, 1959 meeting, 177
committees and representatives, 782
constitution and bylaws, 783
editorial boards, 782
members, 941
officers, 782
program, 1959 meeting, 655
symposium on biochemical aspects of cell structure and function, 957–965
symposium on topics of current interest in biochemistry, 966–1000
Biological rhythms, symposium, 1232–1240
Biological significance of the fine structure of macromolecules (Joint Session), 739–758
Biologists, experimental—distribution in the U.S., 759
Boyce, W. H. Effects of high calcium intakes on urine—human beings, 1102
Brody, T. M., 1017

Calcium
absorption, factors influencing, 1119
high intake, effects of, 1085
high intake, effects on absorption of other nutrients, 1119
high intake, effects on bones, blood and soft tissues, 1112
high intake, effects on renal function, 1093
high intake, effects on urine, animals, 1096; man, 1102
Campbell, P. A. Progress toward space flight, 1255
Cell walls, bacterial, chemistry of, 966
Cells
biochemical aspects of structure and function, 957
liver, action of carbon tetrachloride on, 1013
membrane as the site of damage by heavy metals, 1036
structure and function, biochemical aspects of, symposium, 957–965
Chang, Min-Chueh, 1051
Clamann, H. G. Some metabolic problems of space flight, 1249
Clarkson, T. W., 1036

Davis, G. K. Effects of high calcium intakes on the absorption of other nutrients, 1119
Deoxyribonucleic acid, genetic activity of, 957
Drill, V. Biological effects of some steroids with progestational activity, 1040

Edsall, J. T. Opening remarks (Joint Session of the Federation), 739
Enzymic oxidation of pharmacologically active amines, 1005
Exhibits, 43rd Annual Meeting
industrial scientific, 724
member and institutional, 735

Federation
Board, 1959–1960, 771
Board, proceedings (1958–1959), 764
committees, 1959–1960, 771
constitution and bylaws, 775
deceased members, 912
financial statement, 770
former executive committees and boards, 771–774
intersociety sessions, 43rd Annual Meeting, 610
Joint Session, 43rd Annual Meeting, 609
meetings, Atlantic City (1959), 607; Chicago (1960), lvi
membership, alphabetical, 800; geographical, 913; by Society, 936
Placement Service, 777
Fenn, W. O. Life in space, symposium, 1241
Fertility-controlling steroids, symposium, 1039–1074

Ganglion-stimulating agents, non-nicotinic, 1001
Garcia, C. R., 1051
Genetic activity of deoxyribonucleic acid in reconstitution of biosynthetic pathways, 957
Green, D. E. and R. L. Lester. Role of Lipides in the Mitochondrial Electron Transport System, 987
Greenblatt, R. B., 1055
György, P. Protein requirement and its assessment in man, symposium, 1125

Habel, Ruth C. and Eleanor M. Olson. Distribution of experimental biologists in the U.S., 759
Hagen, P. B. Enzymic oxidation of pharmacologically active amines, 1005
Harper, A. E. and U. S. Kumta. Amino acid balance and protein requirement, 1136

[1] S refers to pages in Supplement 3, Advances in Human Nutrition (July 1959).

HARRIS, R. S., 1100
HARRISON, H. E. Factors influencing calcium absorption, 1085
HEGSTED, D. M., 1123
HEGSTED, D. M. Protein requirement in man, 1130
Heinz international symposium on nutrition, proceedings, S1-148
HELLER, C. G. Effect of progestins upon human spermatogenesis, 1057
HENNEMAN, P. H. Effects of high calcium intakes on renal function, 1093
Hexoses, interconversion of, 984
HOAGLAND, H. Fertility-controlling steroids, symposium, 1039
HODGE, H. C. Toxicology, symposium, 1013

Immunologists, Association of
abstracts, 1959 meeting, 555
committees and representatives, 797
constitution and bylaws, 797
editorial board, 797
members, 953
officers, 797
program, 1959 meeting, 714
Intersociety Sessions, 43rd Annual Meeting, 610

JAGENDORF, A. Photosynthetic phosphorylation, 974
Joint Session, 43rd Annual Meeting, 609, 739–758
JUDAH, J. D. AND K. R. REES. Mechanism of action of carbon tetrachloride on liver cells, 1013

KENDREW, J. C. Structure and function in myoglobin and other proteins, 740
KING, J. STANTON, JR., 1102
KUMTA, U. S., 1136
KUPPERMAN, H. S., 1071

LAIDLAW, W. M., 1057
LESTER, R. L., 987
LEVENSON, S. M. AND D. M. WATKIN. Protein requirements in injury and certain acute and chronic diseases, 1155

Macromolecules, biological significance of fine structure, 739–758
MCGINTY, D. A., 1048
Metabolic problems of space flight, 1249
MICKELSEN, O. Effects of high calcium intakes, symposium, 708, 1075
Molecular complementarity and antidotes for alkylphosphate poisoning, 752
MOORE, D. J., 1057
Myoglobin and other proteins, structure and function, 740

NELSON, W. O., 1057
Nitrogen content of the body and its relation to retention and loss of nitrogen, 1125
Nucleic acids, properties of polynucleotides and, 987
Nutrition
advances in, Heinz international symposium, S1-148
basic research, S75
education S115
health problems in middle age, S35
protein, and enzyme changes in man, 1143
protein requirement and its assessment in man, 1125–1231
the growing process, S1
Nutrition, Institute of
abstracts, 1959 meeting, 515
committees and representatives, 793
constitution and bylaws, 794
editorial board, 793
members, 951
officers, 793
program, 1959 meeting, 706
symposium on effects of high calcium intakes, 1075–1124
symposium on protein requirement and its assessment in man, 1125–1231

OHLSON, MARGARET A. AND GENEVIEVE STEARNS. Calcium intake of children and adults, 1076
OLSON, ELEANOR M., 759
Organic phosphate poisoning, 1020

Pathology, Society for Experimental
abstracts, 1959 meeting, 465
committees and representatives, 791
constitution and bylaws, 791
members, 949
officers, 791
program, 1959 meeting, 700
PAULSEN, C. A., 1057
Pharmacology and Experimental Therapeutics, Society for,
abstracts, 1959 meeting, 361
committees and representatives, 787
constitution and bylaws, 788
editorial boards, 787
members, 947
officers, 787
program, 1959 meeting, 683
symposium on autonomic pharmacology, 1001–1012
symposium on fertility-controlling steroids, 1039–1074
symposium on toxicology, 1013–1038
Phosphate poisoning, organic, 1020
Phosphorylation, photosynthetic, 974
Physiological Society
abstracts, 1959 meeting, 1
committees and representatives, 778
constitution and bylaws, 779
editorial boards, 778
members, 936
officers, 778
program, 1959 meeting, 628
symposium on biological rhythms, 1232–1240
symposium on life in space, 1241–1256
PINCUS, G., J. ROCK, MIN-CHUEH CHANG AND C. R. GARCIA. Effects of certain 19-nor steroids on reproductive processes and fertility, 1051
Polynucleotides and nucleic acids, properties of, 987
Progestins
effect on reproductive physiology of normal men, 1057

in gynecology and obstetrics, 1066
steroids with progestational activity, 1040
Protein
calories and life expectancy, 1190
malnutrition and infection, 1207
myoglobin and others, structure and function, 740
nutrition and enzyme changes in man, 1143
requirement and amino acid balance, 1136
requirement in injury and certain acute and chronic diseases, 1155
reserve and nitrogen content of the body, 1125
structure and function, myoglobin and other, 740
Protein requirement and its assessment in man, symposium, 1125–1231

Radiation hazards in space flight, 1242
RAKOFF, A. E. Progestational compounds in gynecology and obstetrics, 1066
REES, K. R., 1013
Renal function, effects of high calcium intakes on, 1093
ROCK, J., 1051
ROSEMAN, S. Interconversion of hexoses, 984
ROSS, M. H. Protein, calories and life expectancy, 1190
ROTHSTEIN, A. Cell membrane as site of damage by heavy metals, 1026

SCRIMSHAW, N. S. Protein malnutrition and infection, 1207
Space flight
metabolic problems, 1249
progress toward, 1255
radiation hazards, 1242
Space, life in, symposium, 1241–1256
SPIZIZEN, J. Genetic activity of deoxyribonucleic acid in the reconstitution of biosynthetic pathways, 957
STEARNS, GENEVIEVE, 1076
Steroids, fertility-controlling, symposium, 1039–1074
Symposia and special sessions, 1959 meeting
autonomic pharmacology, 683, 1001–1012
biochemical aspects of cell structure and function, 655, 957–965
biological rhythms, 643, 1232–1240
effects of high calcium intakes, 708, 1075–1124
fertility-controlling steroids, 687, 1039–1074
life in space, 649, 1241–1256
protein requirement and its assessment in man, 710, 1125–1231
topics of current interest in biochemistry, 674, 966–1000
toxicology, 686, 1013–1038

TOBIAS, C. A. Radiation and life in space, 1242
Toxicology alkylphosphate, molecular complementarity and antidotes for, 752
Toxicology, symposium, 1013
TRENDELENBURG, U. G. Non-nicotinic ganglion-stimulating agents, 1001

VERMEULEN, C. W. Effects of high calcium intakes on urine—animals, 1096

WALLACE, W. M. Nitrogen content of body and its relation to retention and loss of nitrogen, 1125
WATERLOW, J. C. Protein nutrition and enzyme changes in man, 1143
WATKIN, D. M., 1155
WHEDON, G. D. Effects of high calcium intakes on bones, blood and soft tissues, 1112
WILLS, H. Organic phosphate poisoning, 1020
WILSON, I. B. Molecular complementarity and antidotes for alkylphosphate poisoning, 752

ZILLIKEN, F. Chemistry of bacterial cell walls, 966